Development of an eDNA Approach as a Novel Technique for the Early Detection of Aquatic Fungal Diseases-Causing Agents in the Environment

Report to the
Water Research Commission

by

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EXECUTIVE SUMMARY

BACKGROUND

Human activities, climate change, and emerging infectious diseases are just a few current discussion topics worldwide; each presenting a great variety of challenges. With the aim of improving conservation globally, topics such as these have gained significant research momentum over the last two decades. Conservation efforts largely depend on the ability to assess biodiversity and the health thereof. Such assessments have historically relied on the physical identification of species, using either visual surveys or other traditional techniques. However, these traditional monitoring techniques remain challenging for a number of reasons: they are labour intensive, demanding on resources, there is a lack of standardised sampling methods, there are many difficulties with correctly identifying species, and some sampling techniques are invasive in nature. Environmental DNA offers an alternative to labour-intensive and invasive sampling; but still requires optimisation, validation, and standardisation. Environmental DNA monitoring could be applied as a targeted assay; where the presence of a target species is assessed using species-specific primers, or by using a more general approach in which entire communities are characterised through DNA barcoding.

Emerging infectious diseases continue to pose a threat to the environment; and with animal trade increasing on a global scale, this issue has become even more concerning. The World Organisation for Animal Health has compiled a list of notifiable diseases, with the aim of reducing the risk of these diseases crossing geographical borders and entering non-infected zones. While some of these diseases are easy to screen for, others can be challenging due to the host habitat and the nature of traditional detection techniques.

AIMS

This study aimed to develop an eDNA assay that will serve as the framework of eDNA assays to follow, in both South African and international fresh waters. To achieve the main objective, this study focused on two fungal-like pathogens as model organisms, with the following aims.

1. Develop and optimise a reliable TaqMan probe assay to detect *Aphanomyces invadans.*
2. Develop a field-based sampling protocol to extract DNA from freshwater bodies, for the purpose of molecular analyses.
3. Achieve laboratory validation of a targeted eDNA approach to detect *Batrachochytrium dendrobatidis* and *A. invadans.*
4. Validate a targeted eDNA approach to detect *B. dendrobatidis* and *A. invadans* from aquatic environmental samples in the field.

METHODOLOGY

An assay to sample eDNA and detect *B. dendrobatidis* was designed, optimised, and validated. Each step was assessed independently. Three different sampling methods were compared, and a decision was made based on efficiency and practicality. Preservation mediums and sampling processes were tested. Two crude DNA extractions were tested and compared to two commercial extraction kits. A published molecular assay was used to determine the most efficient option for each step. The novel assay was validated on field samples, by comparing it to one of the gold-standard detection techniques. In addition, a different molecular assay, namely a LAMP assay, was designed for the detection of *B. dendrobatidis.* This is a simplified molecular assay that could serve as a more cost-effective alternative.

Due to the lack of a real-time molecular assay to detect *A. invadans* (a notifiable disease listed by the WOAH, and a threat to South African conservation); a molecular assay was designed, specific to *A. invadans.* Synthetic

DNA and total genomic DNA from environmental isolates were used to determine the analytical specificity and sensitivity of the assay.

RESULTS AND DISCUSSION

The most effective method in each step was chosen to construct the workflow necessary to the detection of *B. dendrobatidis* in the environment. This workflow was validated with field samples. To collect water samples in the field, a syphon pump proved to be cost-effective and efficient. It is also light-weight and easy to carry to remote areas. One preservation buffer was chosen as the medium to preserve filters until processing. The Qiagen DNeasy Powersoil Pro kit was the most effective extraction method, yielding the highest quality and quantity of DNA. While this assay needs a few more validation rounds, it is already showing very promising results, with a LOD of 5 copies/µL And a LOQ of 5000 copies/µL. The LAMP assay development was optimised under laboratory conditions, and the optimal isothermal temperature for this assay was established at 58°C.

The optimal concentrations of primers, and the probe in the qPCR assay to detect *A. invadans*, were determined as 300 nM and 200 nM respectively. The assay specificity was confirmed when none of the other environmental organisms amplified. The LOD was determined as 5 copies/µL, while the LOQ was calculated at 5000 copies/µL. This first-of-its-kind qPCR assay to detect *A. invadans* has an assay efficiency of 99.14%.

CONCLUSIONS

The establishment of a foundational assay to detect eDNA was successfully achieved, and the optimised workflow is ideal for the detection of fungal pathogens. In addition, with minor adaptations (depending on the target species), this assay can easily be applied to any freshwater species.

RECOMMENDATIONS

More field validations will add to the validity of the assay. The assay developed to detect *A. invadans* can be applied to tissue detections as well as eDNA assays, if validated correctly.

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ACRONYMS & ABBREVIATIONS

Ct	Cycle threshold value
CTAB	Cetyltrimethylammonium bromide
CV%	Coefficient of variation percentage
DNA	Deoxyribonucleic acid
eDNA	environmental DNA
EUS	Epizootic ulcerative syndrome
GIS	Geographic information systems
GPA	Glucose Peptone Agar
HHL	Herpetological Health Lab
ITS	Internal transcribed spacer
LAMP	Loop-mediated isothermal amplification
LOD	Lowest Limit of Detection
LOQ	Lowest Level of Quantification
PCR	Polymerase Chain Reaction
PDA	Potato Dextrose Agar
qPCR	Quantitative Polymerase Chain Reaction
SD	Standard Deviation
WOAH	World Organisation for Animal Health

CHAPTER 1: INTRODUCTION

1.1 INTRODUCTION

Infectious diseases have for many years been a threat to humans, animals, and plants. They are among the leading causes of death and species extinction worldwide; with roots of established infections, as well as epidemics of new and old infectious diseases emerging periodically. Studies of these emerging infections reveal the evolutionary properties of pathogenic microorganisms; and the dynamic relationships between microorganisms, their hosts, and the environment (Morens *et al.*, 2004). Diseases and changes in environmental conditions often create biological stress in the hosts, leaving them immunocompromised and more susceptible to pathogens. To control diseases and disease outbreaks, it is of utmost importance to have a full understanding of the ecology and species present in an identified environment.

Infectious fungal-like diseases are no different. They have also been known threats to a variety of plant species, and more recently to terrestrial and aquatic animals (Fisher *et al.*, 2012). Over the last two decades, there has been an increased threat, specifically to food security. Records of disease epidemics in plants caused by fungi and fungal-like oomycetes date back to the nineteenth century; when late blight led to starvation, economic ruin, and the downfall of the English government during the Irish potato famine. In the twentieth century, Dutch elm and chestnut blight were recorded (Fisher *et al.*, 2012). Despite these records, fungal infections have still largely been underestimated. In addition, it has left significant footprints in many conservation areas, such as: White-nose syndrome in bats (caused by *Geomyces destructans*), Chytridiomycosis in amphibian species (caused by *B. dendrobatidis*), sea-fan aspergillosis in soft corals (caused by *Aspergillus sydowii*), colony collapse disorders in bees (caused by *Nosema* sp.), crayfish plague (caused by Aphanomyces astaci), Epizootic ulcerative syndrome in a variety of fish species (caused by *A. invadans*), and *Fusarium* infections in sea turtle eggs (Fisher *et al.*, 2012; Peeler *et al.*, 2015; Iberahim *et al.*, 2018b; Scheele *et al.*, 2019; Greeff-Laubscher & Jacobs, 2022).

Most fungi and fungal-like organisms can survive outside their hosts, either as free-living saprophytes or durable spores in the environment; increasing both their ability to survive in severe environmental conditions, and the possibility of finding a susceptible host. Pathogenic fungi with a saprophytic stage can lead to host extirpation, due to their growth rate being independent of host densities, while many fungal diseases threatening natural populations are caused by opportunistic fungi with long-lived environmental stages. Infectious fungal-like diseases have long been a known threat to a variety of plant species, but have also become an increasing threat to both terrestrial and aquatic animals (Fisher *et al.*, 2012). Diseases and changes in environmental conditions often create biological stress in the hosts, leaving them immunocompromised and more susceptible to pathogens.

1.1.1 *Batrachochytrium dendrobatidis*

The agent responsible for chytridiomycosis in amphibians, *Batrachochytrium dendrobatidis* is a basal fungal lineage in the division Chytridiomycota. Due to the unique structural and genomic traits of *B. dendrobatidis*, it has been placed as the lone member of its genus within the order Rhizophydiales (Fisher *et al.*, 2009). The first record of *B. dendrobatidis* infection dates to the 1930s. More specifically, in South Africa, the *B. dendrobatidis* infection was first reported in the endemic and critically endangered Cape Platanna, *Xenopus gilli*, in 1938. *Batrachochytrium dendrobatidis* has been recognised globally for contributing to the decline of 501 amphibian species, of which 90 are now presumed extinct (Tarrant *et al.*, 2013, Scheele *et al.*, 2019). One of the first hypotheses on the geographic origin and spread of

B. dendrobatidis, suggested that it originated in and spread from Africa via the global trade in African clawed frogs (*Xenopus laevis*) (Weldon *et al.*, 2004); and the link between the amphibian trade pathway and disease emergence has been assessed for more than one species (Fisher & Garner, 2007, Weldon *et al.*, 2007, Schloegel *et al.*, 2009). This hypothesis was later challenged, based on genetic diversity that pointed to Asia as the most likely origin. Now *B. dendrobatidis* is known to be phylogenetically diverse, consisting of lineages with noticeable variation in virulence, and with distinct geographical ranges. The Global Panzootic lineage (associated with amphibian population declines) also overlaps with some of the other lineages that have more restricted distributions (Farrer *et al.*, 2011, Rosenblum *et al.*, 2013, O'Hanlon *et al.*, 2018). Befitting a true panzootic, *B. dendrobatidis* can readily spread across geographical borders, given its wide host range, and its ability (albeit limited) to survive outside the host (Johnson & Speare, 2003, Weldon & Fisher, 2011).

The chytrid fungus grows in the keratinised layer of the epidermis – the stratum corneum of metamorphosed frogs, concentrated in the region of the hind legs and pelvic patch, and in the keratinised mouthparts of tadpoles. Isolates of *B. dendrobatidis* can grow at 4-25ºC and at pH 4-8, and experience mortality when temperatures rise above 30ºC (Longcore & Pessier, 1999). The life cycle takes about 4-5 days to complete, when grown in pure culture (Longcore & Pessier, 1999). The infective stage of the chytrid fungus is a motile zoospore that can either reinfect the same individual or transmit to other hosts when released into water. After locating a susceptible animal, zoospores penetrate the skin and develop into spherical mature zoosporangia with discharge tubes. Zoospores are produced inside the sporangia. The zoospores are released through the discharge papillae. Sometimes thin septa form within the sporangium, that divide the contents into two or more compartments, each with its own discharge papilla. Such a zoosporangium is referred to as a colonial thallus. After the zoospores have been released, all that is left are empty sporangia that often become colonised by bacteria.

Amphibians that have contracted the chytrid fungus and are susceptible to disease can be identified, and the condition described in terms of clinical symptoms and gross and microscopic lesions. Although the pathology and the clinical signs of chytridiomycosis are similar in amphibians, the extent of clinical signs varies greatly depending on the species of amphibian host. Chytridiomycosis manifests as clinical symptoms related to the central nervous system, including: abnormal behaviour and body posture, e.g. nocturnal frogs squatting unprotected during the day, with limbs in an unusual position away from the body; lethargy and loss of righting reflex; and sloughing of the skin (Berger *et al.*, 1999, Daszak *et al.*, 1999). Chytridiomycosis has a clinical course of around three weeks, resulting in the death of the infected host. Gross lesions are often subtle, and usually restricted to the legs and ventrum of post-metamorphic amphibians; and may vary from mild skin thickening and discolouration, to swollen limbs and slight reddening of the skin (Nichols *et al.*, 2001, Bradley *et al.*, 2002). Infected tadpoles often have discoloured rostrodonts (jaw sheaths) or missing keratodonts (teeth rows), despite a healthy appearance (Lips, 1999, Fellers *et al.*, 2001, Lips *et al.*, 2004). Epidermal tissue at the area of infection usually develops hyperkeratosis and hyperplasia (thickening of the stratum corneum), and could include intercellular edema and ulceration; while inflammatory cell response is almost absent (Berger *et al.*, 1998, Nichols *et al.*, 2001, Bradley *et al.*, 2002, Lane *et al.*, 2003). It appears that amphibians that do not die from the infection, do not consistently show a thickening of the outer epidermal layer; or else show a mild response (Daszak *et al.*, 2004, Hanselmann *et al.*, 2004). Host death is ultimately a result of a disruption in the physiological functions of amphibian skin (Voyles *et al.*, 2009). The dense aggregation of fungal thalli in the epidermis impairs fluid and electrolyte homeostasis, respiration, and the skin's role as a barrier to toxic and infectious agents.

Global mapping has enabled an overview of the chytrid panzootic: *B. dendrobatidis* has been found infecting 1,015 of 1,854 (54%) species, and at 3,705 of 9,503 (39%) field sites (Fisher & Garner, 2020). In 2014, *B. dendrobatidis* infected 50% of tested frog species (order Anura), 55% of salamander and newt species (clade Caudata), and 29% of caecilian species (Gymnophiona), testifying to an extraordinary and unmatched pathogen host range. A meta-analysis synthesised data from multiple

sources, which included peer-reviewed studies; the International Union for Conservation of Nature (IUCN) Red List of Threatened Species; and consultations with the scientists investigating the declines, both as they occurred and retrospectively (Scheele *et al.*, 2019). This meta-analysis revealed that chytridiomycosis has contributed to the decline of at least 501 species (6.5% of all amphibian species), leading to presumed extinctions; and decreases in abundance exceeding 90% in another 124 species. To date the chytrid panzootic represents the greatest documented loss of biodiversity attributable to a non-human species.

1.1.2 *Aphanomyces invadans*

Aphanomyces invadans is an Oomycete known to be the infectious agent of Epizootic Ulcerative Syndrome (EUS). *Aphanomyces invadans* is one of the most threatening fungal-like pathogens to fish species in South Africa and is listed by the World Organisation of Animal Health (WOAH) as a notifiable disease. This fungus has undergone a few name changes over the years. It was first described and named as *A. invaderis*, after which it was changed to *A. piscicida.* Following that, it changed to EUS-related Aphanomyces (ERA), and eventually to *A. invadans* as we know it today. Similarly, the disease caused by this fungus-like organism also changed names. It was originally referred to as Epizootic granulomatous aphanomycosis (EGA), and then became Epizootic ulcerative syndrome (EUS) (Iberahim *et al.*, 2018a).

Epizootic ulcerative syndrome was first reported in the early 1970s when characteristic lesions were described on farmed Ayu sweetish, *Plecoglossus altivelis* in Japan (Lilley *et al.*, 1998). Roughly four decades later, the first reported EUS outbreak in South Africa was documented in the Western Cape province. Due to the epizootic nature of *A. invadans* and the broad host range, dissemination occurred rapidly to various water systems, both locally and internationally. To date, EUS has been reported by 20 countries globally, occurring in over 100 fish species (Kamilya & Baruah, 2014, World Organisation for Animal Health, 2021). There is no information available to indicate that fish can be lifelong carriers of *A. invadans.* While fish with mild or moderate infections might recover, most fish die during an outbreak (World Organisation for Animal Health, 2021). The spread of this disease holds a significant threat to the fish populations and their direct environments. In addition, EUS poses a threat to the freshwater aquaculture industry; and an outbreak could substantially affect farmers and fishermen, especially in regions where fish serves as the only affordable and reliable protein source for local communities.

Infection with *A. invadans* occurs when environmental conditions favour sporulation, such as water temperatures of 18-22°C, and after heavy rainfalls. *Aphanomyces invadans* follows the typical life cycle of an oomycete, without the sexual stage. The asexual stage in the *Aphanomyces* genus is characterised by the formation of biflagellate zoospores from clusters of primary cysts at hyphal tips. Zoosporangia consisting of 30-50 primary zoospores are formed. Zoospores are then released through a lateral evacuation tube into the environment. After releasing the zoospores, primary zoospores will immediately encyst at the apical tip to form achlyoid clusters. From these clusters, secondary zoospores are released. Later, these secondary zoospores will germinate by forming a germ tube, which eventually develops into mycelium; while encysted zoospores can release new zoospores instead of germinating, a process referred to as repeated zoospores emergence (RZE), or polyplanetism (Diéguez-Uribeondo *et al.*, 1994, Iberahim *et al.*, 2018a). Mycelium is a cylindrical hyphoid and coenocytic. In infected tissue, hyphae can be seen with limited branching and a diameter of up to 27 µm. This diameter is significantly smaller when cultured *in situ.* Under natural conditions, sporulation will occur in waters with a temperature of 25°C and low salinity (0-8 psu) (Kiryu *et al.*, 2005).

Infections are initiated when motile zoospores attach to their host where the skin is damaged. The zoospores germinate and hyphae penetrate the epidermis into the deeper subcutaneous tissue layers;

and into the underlying skeletal muscle, resulting in extensive ulceration and destruction of the infected tissues. The occurrence of skin lesions varies according to the fish species. Clinical signs include lesions varying from red areas of inflammation; to open, ulcerated, necrotic wounds (Mchugh *et al.*, 2014). Histologically, infection is characterised through the observation of deeply penetrating hyphae into muscle tissue, with associated inflammation characterised by lymphocytic infiltration and enclosed granulomas. Haematological data has shown a significant increase in white blood cells; along with a significant decrease in red blood cells, haemoglobin concentration, and the haematocrit level. This is due to the blood loss caused by lesions, leaving fish to suffer from anaemic conditions (Iberahim *et al.*, 2018a).

The epizootic nature and broad host range of *A. invadans* enables these causative agents to spread easily across geographical borders (FAO, 2009, Oidtmann, 2012, Songe *et al.*, 2012). Most African countries recognise their exposure to transboundary aquatic animal diseases, as well as their vulnerability to such diseases. Reasons for this include inadequate policy and legislation, insufficient human infrastructure and institutional capacity, and a lack of specialised diagnostic capacity (Christison, 2019). Infection with *A. invadans* (EUS) has been associated with severe negative biodiversity and social impacts in Africa, such as a significant loss of income for African fishermen and fish farmers alike. Globally it is recognised that infectious diseases such as EUS are a major constraint on the future growth and sustainability of aquaculture production and trade. At the aquaculture establishment level, direct financial costs attributable to infectious diseases include production losses due to mortality, and the cost of veterinary care. Indirectly, elevated financial or production costs attributed to infectious diseases include reduced growth rates of the fish, and increased susceptibility to other secondary infections and environmental stressors. Trade restrictions, particularly on diseases listed with the WOAH such as EUS, are also considered to be significant constraints on sustainable aquaculture development at national and regional levels (Christison, 2019).

1.1.3 Current sampling and detection methods

Several diagnostic methods exist for *B. dendrobatidis.* These include histopathology, histo-chemistry, PCR assays, and electron microscopy (Berger *et al.*, 1998, Hyatt *et al.*, 2007). Previous diagnostics of the pathogen were performed on toe clips of amphibians through histological as well as immune-histochemical examinations (Annis *et al.*, 2004, Boyle *et al.*, 2004). The sporangia of the pathogen can also be observed through microscopy of the mouthparts of tadpoles, or toe clips and skin samples from adult anurans. Toe clipping does, however, cause severe stress to the organism and is not recommended for long-term studies (Hyatt *et al.*, 2007). Swabbing is considered more ethical and is generally done on the ventral side of adults, due to the highest density of infections most commonly occurring around the inner thighs, as well as between the toes of the hind-feet (Annis *et al.*, 2004). In certain fully aquatic species, infection may occur on the ventral as well as dorsal surfaces (Annis *et al.*, 2004). Boyle *et al.* (2004) developed a species-specific diagnostic assay that can be used on DNA extracts from ventral swabs, followed by amplification with a TaqMan probe real-time PCR assay. Although this method is less invasive than the previous diagnostic assays, it still requires interaction with the host specimen, which may potentially cause varying degrees of stress to the individuals.

While current diagnostic assays to detect *A. invadans* do not include a real-time PCR assay, there are other assays recognised by the WOAH to screen fish exhibiting clinical signs. These methods include direct detection of *A. invadans* through: (1) isolation and identification of *A. invadans,* (2) histopathology in combination with confirmation of two molecular techniques, (3) Fluorescent peptide nucleic acid *in-situ* hybridisations, and (4) polymerase chain reaction (PCR) amplification of the DNA of *A. invadans* (OIE, 2018). All these diagnostic methods have been, and still are used with great success.

However, each of these methods for both species in question have shortcomings; the most common is their dependence on visible clinical signs. Catching hosts with visible clinical signs can be time-consuming, costly, and often missed; which has a negative impact on the sensitivity of the assay. As an example, early infections and asymptomatic hosts can easily be missed, leading to an infected host remaining undiagnosed. This leads to the underestimation of disease prevalence, and an increased risk of carrier hosts spreading the disease, putting healthy populations at risk. To overcome these challenges a method is required to detect the infectious agent in the environment without host interaction, even when present in low concentrations or asymptomatic host populations.

1.1.4 Environmental DNA

Environmental DNA (eDNA) analyses could assist in early detection and the understanding of species distribution to inform risk assessments and targeted surveillance efforts.

Environmental DNA can be described as DNA fragments that are derived from scales, metabolic waste, deceased organisms, skin, or other bodily excrements that can be sampled from the abiotic environment (Seymour *et al.*, 2018). The use of eDNA has gained significant interest over the last decade, especially for its application in biodiversity and ecological studies. This is due to the simplification of sampling and sample processing, allowing for rapid processing of large sample numbers; and thus making more it cost and time effective. (Huver *et al.*, 2015, Darling *et al.*, 2017, Harper *et al.*, 2019). Environmental DNA, in conjunction with species-specific DNA amplification, could assist in the early detection of species, and in addition assist in the understanding of species distribution in time and space (Moyer *et al.*, 2014, Darling *et al.*, 2017). The basic principle includes filtering, preservation, and extraction of the DNA from the environment, followed by amplification of DNA fragments, either targeted or non-targeted (Deiner *et al.*, 2015).

Many species present within the environment cannot always be observed with the naked eye or may be missed during field surveying, especially rare or cryptic species (Jerde *et al.*, 2011, Schmidt *et al.*, 2013). In the application of eDNA detection, species in lower numbers or species that are difficult to identify can be found in a specific area, which might otherwise have been missed through conventional survey methods (Dejean *et al.*, 2012). Environmental DNA assays reduce these errors by analysing the DNA or cells within aquatic environments, and do not require the host specimen to be present at the time of sampling (Schmidt *et al.*, 2013).

1.1.4.1 Sampling methods for eDNA

Sampling for eDNA is the first step of the assay. While this seems to be a simple step, it is a step with many possibilities and variables that cannot be controlled, such as water turbidity and flow rate. Sampling methods used in any eDNA study play a crucial role in the successful detection of eDNA. Dispersion and degradation of samples during fieldwork significantly influences successful detection. Sampling techniques must often be adapted for eDNA, based on the type of environment being sampled (Kamoroff & Goldberg, 2017). Two sampling techniques can be applied for the sampling of eDNA; namely, filtration and precipitation (Deiner *et al.*, 2015, Goldberg *et al.*, 2018). Filtration requires a much larger volume of water than precipitation (Deiner *et al.*, 2015). The filtration methods tend to be more successful than precipitation, although the difference between the results is not significant (Deiner *et al.*, 2015).

Filtration methods often vary between studies, and can be applied to a diverse range of uses, while several variables can be altered to fit each selected use's preference. Different pore sizes, along with various filter materials; such as cellulose nitrate, glass, and nylon fibres can be applied (Deiner *et al.*, 2015, Huver *et al.*, 2015, Agersnap *et al.*, 2017, Goldberg *et al.*, 2018). Whatman cellulose nitrate

membranes with different pore sizes, for example, were used in the study by Huver *et al.* (2015). They reported that pore sizes of 0.2 µm often resulted in clogging, and that only less than 250 mL of water could be filtered at a given time. However, smaller pore sizes are more effective in collecting eDNA than larger pore sizes. It was determined that 3 µm pore sizes were the most effective for collecting the DNA, while 500 mL of water could be sampled per session (Huver *et al.*, 2015). However, in the study by Agersnap *et al.* (2017), pore sizes of 0.22 µm were applied, and they were able to sample a larger volume of water, i.e., 0.5-1.5 L per session. This could possibility have been due to differences between the two aquatic environments. Agersnap *et al.* (2017) explained that turbidity influences the amount of water that can be sampled before clogging occurs. Smaller pore sizes can present certain constraints in filtration; but if the pore sizes are too large, samples may be lost (Goldberg *et al.*, 2018). When applying different filtering mechanisms, it is very important to consider the water conditions when selecting materials and pore sizes for effective sampling.

Filtration materials should also be replaced for every sampling run, to prevent any cross-contamination occurring between sites (Huver *et al.*, 2015). Agersnap et al. (2017) recommended extracting DNA less than twenty-four hours after filtering to minimize the degradation of the sample. Filtrations can be done on-site, or water samples can be brought to the lab for filtering (Deiner *et al.*, 2015). When filtering is conducted in a lab it is recommended to use a laminar flow cabinet to limit contamination (Deiner *et al.*, 2015). Filtering in the field, rather than bringing water samples to the laboratory, could increase accuracy. All the above variables need to be considered when developing a sampling method. The main aim of this study is to develop a novel method that can be applied in South Africa to detect fungal pathogens.

1.1.4.2 Sample processing

Extracting eDNA from filters is another step to be optimised and validated for the intended application. In this study it is likely that fungal spores will be present on the filters, thus an extraction method is needed that can successfully break down the cell walls of spores, as well as extract DNA efficiently when present in low quantities.

Several eDNA extraction protocols exist that cover both main routes of DNA extraction – crude methods and extraction kits. Crude methods are easy and simple to conduct using basic molecular laboratory reagents. It is economical and the steps are easily amendable to manipulate the desired outcome. Two of the better known crude extraction methods are Salting out and Phenol chloroform (Chi *et al.*, 2009). DNA extraction kits are often preferred over crude extraction methods, due to chloroform and phenol being toxic (Barbier *et al.*, 2019). The protocols for crude methods can be time-consuming to perform, while a kit can yield high quality DNA within a shorter time frame (Barbier *et al.*, 2019). Although chloroform and phenol are extremely popular for DNA extractions, they are less effective when used on DNA with low copy numbers. This can prove troublesome, since this phenomenon of low copy numbers is often observed in research for chytrid (Adams *et al.*, 2015). Environmental DNA tends to have lower copy numbers overall than most other DNA, due to more degradation from environmental stressors. For the current study we will compare both kit extractions and crude extraction methods.

The list of available DNA extraction kits is endless, and kits are mostly chosen based on the quantity and quality of required DNA. Some DNA kits, such as Omega Bio-tek kits, have shown in previous literature to deliver high yields of DNA, but a lower quality of these molecules. Qiagen kits will generally provide a lower quantity of DNA, but a higher quality level. If good DNA quality can be achieved, more accurate results can be expected during amplification and sequencing. Beads are frequently used in molecular research and have shown to deliver results with a higher accuracy than some generally manufactured kits. The following methods have previously been applied in chytrid studies: DNAeasy and Zymo research.

DNeasy was often used in previous studies for eDNA extractions (Agersnap *et al.*, 2017, Goldberg *et al.*, 2018). The assays followed for this method are generally provided by the manufacturers, and adjustments are made depending on the study and the intended application (Deiner *et al.*, 2015). A variety of Qiagen kits exist for different extraction purposes. However, the most common kit that was applied in previous studies for chytrid is the Qiagen DNeasy Blood and Tissue kit (Deiner *et al.*, 2015, Kolby *et al.*, 2015, Piggott, 2016, Goldberg *et al.*, 2018).

Zymo Research kits used in a study focussing on fungal spores delivered both high quality and quantities of DNA. Although this method has not often been utilised in previous chytrid studies, it may potentially deliver better results. Du Preez (2019) compared a Dneasy Blood and Tissue kit to a Zymo Research kit in the extraction of *B. dendrobatidis* DNA. The Zymo Research kit yielded both higher quality and quantity DNA than the DNeasy kit – this was after an additional heating step was included to aid in cell wall disruption.

1.1.4.3 DNA amplification

The success of an eDNA assay is partly dependant on the efficiency of the molecular assay used to amplify low quantities of eDNA from the environment. For many eDNA studies, marker genes are applied, and the products are sequenced to determine the species present. However, species-specific primers can also be developed to only amplify the target organism through real-time PCR (Stoeckle *et al.*, 2018). The ITS regions of rDNA have previously been applied successfully to detecting eDNA in fungal species within aquatic environments. This is due to this region of the genome being conservative among the lineages (O'Hanlon *et al.*, 2018). The ITS-1 and ITS-2 regions are popular for the diagnostic purposes of *B. dendrobatidis* (Hyatt *et al.*, 2007). These regions do not vary significantly between the different lineages, and species-specific primers have been developed for PCR as well as qPCR assays (Annis *et al.*, 2004, Boyle *et al.*, 2004). The ITS-1 region is an ideal primer/probe site for specific fungal diagnostics, because it occurs more than 100 times within the genome that creates a multitude of binding sites for amplification (Boyle *et al.*, 2004, Longo *et al.*, 2013). This aids in increasing the sensitivity of the protocol.

1.1.5 Targeted approach to detecting environmental DNA

1.1.5.1 Batrachochytrium dendrobatidis

Two different primer sites have been identified with primers designed for *B. dendrobatidis*-specific studies (Annis *et al.*, 2004, Boyle *et al.*, 2004). The protocol developed by Boyle *et al.* (2004) uses a TaqMan Probe qPCR assay. The primers used for the Taqman assay by Boyle *et al.* (2004) targeting the ITS 1 and 5.8S region, have been used with great success. The forward primer ITS 1- Chytr 3 (5'-CCTTGATATAATACAGTGTGCCATATGTC-3') and Taqman probe MGB2 (5'-6FAM CGAGTCGAACAAAAT MGBNFQ-3') located within the ITS-1 region, are perfectly positioned to exclude the G-rich stretch as well as the AT-rich loop structure. The reverse primer 5.8 Chytr (5'-AGCCAAGAGATCCGTTGTCAAA-3') is located within the 5.8S region immediately adjacent to the ITS-1/5.8S junction. According to Boyle et al. (2004), the TaqMan assay can detect *B. dendrobatidis* in amphibians with the presence of only one single zoospore, although high levels of variability were seen at the lower concentrations in multiple studies (Boyle *et al.*, 2004, Blooi *et al.*, 2013). This could be very promising for eDNA analysis due to the qPCR assay being able to function on limited samples with low quantities of DNA present. The specificity was confirmed by screening with three different *B. dendrobatidis* strains and five other species from the order Chytridiales, where only the *B. dendrobatidis* strains amplified (Boyle *et al.*, 2004). Additional studies further examined the primers, and results showed a high level of specificity when testing various other orders and species in the phylum Chytridiomycota (Hyatt *et al.*, 2007, Blooi *et al.*, 2013).

The second assay developed was by Annis *et al.* (2004) and applies a single round of conventional PCR on the 5.6S ribosomal RNA. The primers from Annis *et al.* (2004) target the ITS-1 and ITS-2 region of the genome. The primers used by Annis *et al.* (2004), often applied in chytrid studies, are known as the Bd1a (5'- GAGTGTGCCATATGTCACG-3') and Bd2a (5'- CATGGTTCATATCTGTCCAG-3') primers. This assay has the lowest detection limit of approximately 10 zoospores; however, 1 zoospore could occasionally be detected (Annis *et al.*, 2004). The specificity of this assay was confirmed using different *B. dendrobatidis* isolates, other closely related species, species from the order Chytridiales, as well as other fungi that may commonly be found in the environment (Annis *et al.*, 2004). The TaqMan Probe assay showed a higher level of sensitivity compared to the conventional PCR method (Goka *et al.*, 2009). However, the primers by Annis *et al.* (2004) were applied using a nested PCR assay and a significant increase in sensitivity was detected (Goka *et al.*, 2009).

1.1.5.2 Aphanomyces invadans

Current molecular techniques to detect *A. invadans* DNA from tissue samples include three assays. The first assay is a species-specific primer set from Vandersea *et al.* (2006), with the forward primer site located near the 3' end of the small subunit gene; a species-specific reverse primer site located in the ITS1 region for Ainvad-2F (5'-TCA-TTG-TGA-GTG-AAA-CGG-TG-3'); and Ainvad-ITSR1 (5'-GGC-TAA-GGT-TTC-AGT-ATG-TAG-3'), amplifying 234bp. The second and third assay are both targeting the ITS1 and ITS2 regions. The assay described by Phadee *et al.* (2004) amplifies a final product of 55 bp, using a forward primer ITS11 (5'-GCC-GAA-GTT-TCG-CAA-GAA-AC-3') along with the reverse primer ITS23 (5'-CGT-ATA-GAC-ACA-AGC-ACA-CCA-3'). The third assay makes use of the primer set BO73 (5'-CTT-GTG-CTG-AGC-TCA-CAC-TC-3') and BO639 (5'-ACA-CCA-GAT-TAC-ACT-ATC-TC-3') to amplify 564bp (Oidtmann *et al.*, 2008). All three of these primer sets have proved to be specific and sensitive when isolating DNA from tissue samples. However, due to the short and fragmented DNA extracted during eDNA sampling, a Taqman probe is required to amplify even shorter DNA fragments than the current assays. This quantitative assay makes use of two primers to amplify a small section of target DNA. Nested within the two primers is a probe which is labelled with a fluourescent reporter dye on one end and quencher molecular on the other end. The probe will cleave during the extension phase of the polymerase reaction, leading to the separation of the quencher, which in turn leads to an increase in flourescent signal. As a result, the level of fluourescence is proportional to the quantity of target DNA (Wilcox *et al.*, 2013). This is a highly specific and sensitive molecular method that will enable the detection of specific target environmental DNA.

1.1.5.3 Host species

Species of the host genus *Amietia* were selected as an assay internal control for the detection of *B. dendrobatidis.* The river frog, (*Amietia delalandii*) is one of many species in South Africa susceptible to *B. dendrobatidis.* The river frog has a wide distribution, specifically in areas where we expect to find *B. dendrobatids* and where *B. dendrobatids* has previously been recorded.

1.1.6 Validation of a diagnostic assay

Diagnostic assays must perform consistently and reliably over time to reduce the inevitable variability introduced by the application of these assays by different operators; often from different laboratories, who make use of varying analyte matrices that may differ in terms of sample origin and quality (Purcell *et al.*, 2011). Therefore, sufficient validation of any diagnostic assay is required before its application; thereby preventing the spread of disease through aquatic animal movement and trade. This in turn contributes to the following: eradication of infection; confirming diagnosis in clinical cases; estimating infection prevalence, facilitating risk analysis; identifying infected animals, enabling implementation of control measures; and classifying animals for herd health, or immune status post-vaccination. The

WOAH defines validation as a “process that determines the fitness of an assay (diagnostic test), which has been properly developed, optimised and standardised for an intended purpose”. Assay validation includes estimates of the analytical and diagnostic performance characteristics of a test. Investing in validating the assay for its intended purpose before working with valuable samples will save time and expense, and will also help to avoid failed runs or inconsistent experimental data (Bustin & Huggett, 2017). Primers and probes used are arguably the single most critical components of any qPCR or PCR assay, as their properties control exquisite specificity and sensitivity that make this application uniquely successful. It therefore follows that poor design, in combination with failure to optimise reaction conditions, is likely to result in reduced technical precision, and false positive or negative detection of amplification targets (Bustin & Huggett, 2017).

1.1.6.1 Assay optimisation

The thermodynamic stability of a duplexed primer/target structure differs for different primers and varies with primer concentration. Therefore, it is important to use primers at concentrations that result in optimal hybridization and priming. The effects of varying primer concentrations can differ dramatically between different primer pairs (Nolan *et al.*, 2006). Amplification efficiency is determined by generating a standard curve using serial dilutions of a template and determining the slope from the linear regression of a plot of Ct vs log of the template concentration. If perfect doubling occurs with each amplification cycle, the spacing of the fluorescent curves will be consistent with an approximate value increase of 3.32 cycles for each ten-fold dilution. An acceptable evaluation of PCR efficiency requires a minimum of three replicates, and ideally five orders of magnitude of template concentration. An assay with a 100% efficiency will reflect a slope of -3.32 (Bustin & Huggett, 2017).

1.1.6.2 Analytical specificity

Analytical specificity is the assay’s ability to distinguish the target genomic sequence from non-target sequences, including matrix components (OIE, 2017); and can be further divided into the following sections:

Analytical selectivity
Selectivity refers to the extent to which a method can accurately quantify the targeted analyte in the presence of other components of similar behaviour (Vessman *et al.*, 2001). In other words, this is an analysis of the impact of inhibitors, such as matrix components, on the analytical specificity of the assay. This study aimed to conduct selectivity assessments according to the validation framework of Hiney and Smith (1998); where the analytical performance of the assay is evaluated across various levels of experimental complexity, ranging from a sterile matrix to non-sterile field samples.

Exclusivity
Exclusivity is the capacity of the assay to detect a genomic sequence that is unique to a targeted organism and excludes all other known organisms that are potentially cross-reactive. The nuclear ribosomal internal transcribed spacer region (ITS1-5.8-ITS2) is known as a universal DNA barcode marker for fungi and fungus-like organisms (Schoch *et al.*, 2012a). Previous studies have shown that analyses of the internal transcribed spacer (ITS) regions of rDNA provide a useful means of differentiating species of the related genera *Saprolegnia* and *Achlya* (Lilley *et al.*, 2003a, Greeff-Laubscher *et al.*, 2019). Consequently, available sequence data of conspecific, congeneric and other closely related species are available for desktop evaluation of the exclusivity of the designed probe set.

I*nclusivity*
Inclusivity is the capacity of an assay to detect several strains of a species. It characterises the scope of action for a screening assay. There are four known lineages of chytrid namely *Bd* Global Panzootic

Lineage (*Bd*GPL), *Bd*CAPE, *Bd*ASIA (includes *Bd*CH, Swiss lineage), and *Bd*ASIA/Brazil (O'Hanlon *et al.*, 2018). To date, only one genotype for *Aphanomyces invadans* has been recorded (Lilley *et al.*, 2003a, Diéguez-Uribeondo *et al.*, 2009, Iberahim *et al.*, 2018b).

1.1.6.3 Analytical sensitivity

Analytical sensitivity represents the smallest amount of the analyte that can be measured in a biological sample. The Limit of Detection (LOD) is the estimated amount of the target in a specified matrix that would produce a positive result for at least a specified percentage of the time (OIE, 2017). When using qPCR for quantitation in addition to the identification of the target, a standard curve is generated from known quantities of the target, and is a requirement under the guidelines for the "Minimum Information for Publication of Quantitative Real-Time PCR Experiments" (MIQE) (Bustin, 2010); and for assessing PCR performance. Synthetic oligonucleotides (Conte *et al.*, 2018) such as gBlocks® Gene Fragments (Integrated DNA Technologies) provide an affordable and easily obtainable reference standard for the quantitation of the target analyte, through calibration of the standard curve.

1.2 PROJECT AIMS

The project had the following aims:

1. Develop and optimise a reliable TaqMan probe assay to detect *Aphanomyces invadans.*
2. Develop a field-based protocol to sample from freshwater bodies to extract DNA for molecular analyses.
3. Achieve laboratory validation of a targeted eDNA approach to detect *Batrachochytrium dendrobatidis* and *Aphanomyces invadans.*
4. Validate a targeted eDNA approach to detect *B. dendrobatidis* and *A. invadans* from aquatic environmental samples in the field.

1.3 SCOPE AND LIMITATIONS

While this project focused on two fungal pathogens, the application of the detection assays developed during this assay is very broad. This project aimed to develop an eDNA assay that can be used on the African continent; where the quality of water can be poor, and water bodies are often in remote areas and difficult to access. To save on resources it was decided to optimise and validate each step of the assay under laboratory conditions prior to field sampling. A short popular article was published in the Water Wheel July/August 2020 (Appendix A).

Most filters require a source of power, whether it be electrical, or battery powered, making it challenging to filter water in remote areas. Although water can be collected in containers and filtered at a later stage if required, it is not recommended due to the risk of DNA loss. It has been recommended that if water is sampled in containers, they be centrifuged or mixed prior to filtering to increase the number of zoospores captured (Hyatt *et al.*, 2007). The filtration method used varies between studies, depending on the target organism and the filter pore size required. Smaller filter sizes collect higher concentrations of eDNA, but increase the time required for filtering due to clogging occurring much quicker and water filtering more slowly through the material (Lacoursière-Roussel *et al.*, 2016, Barnes *et al.*, 2021). Finding the appropriate ratio between pore size and filtration time is key to improving the effectiveness of the protocol. The pore size depends on the size of the target organism.

Environmental DNA assays can provide valuable insights into the presence of certain species, but false negatives and positives have also been reported in previous studies. Cross-contamination can occur between sites if the equipment being used is not properly decontaminated prior to each sampling (Goldberg *et al.*, 2016). The workflow of samples from processing to amplification should be carefully considered, with the aim of keeping samples separate during each stage of the assay. Setting standards will limit the possibility of false positives and negatives.

The environment will always have many natural PCR inhibitors, such as humic substances, sediment, and algae. The success of an eDNA assay is dependent on the ability to extract pure nucleic acid, free of any inhibitors, in order to amplify target regions through PCR. Therefore, even if a DNA extraction performs well on a sterile culture, it might be different for environmental samples. Some extraction methods might perform better than others, depending on the sample types and inhibitors present in the sample. Another important factor to consider is the rate at which DNA degrades in the environment. Understanding the target organism and its host can aid in identifying possible limiting factors; for example, the type of marker gene that is used is affected by the degradation rate of DNA.

In the case of fungal pathogens, the ITS region is the barcode gene. When applying the current assay to other organisms, it is important to ensure that the correct gene region is being targeted for optimal success.

Finally, to develop, optimise and validate a molecular based assay, many challenges and limitations must be considered. Unfortunately, molecular research is very costly, as special equipment and expensive reagents and consumables are required; and it is therefore important to take extra care to reduce the risk of contamination.

CHAPTER 2: OPTIMISATION OF SAMPLE PROCESSING

2.1 INTRODUCTION

The optimisation of assays under laboratory conditions prior to collecting field samples is essential to 1) ensure that samples will be processed under optimal conditions, and 2) determine the limitations of the assay. Without knowing the Limit of detection (LOD) or Limit of quantification (LOQ), it is not possible to interpret field results (Piggott, 2016). Following sample collection through filtration, the next challenge is to extract DNA from the filter media (Deiner *et al.*, 2015). Although many different DNA extraction methods have been tested on fungi, results will always vary based on the type of species being used (Fredricks *et al.*, 2005). Environmental factors can also significantly contribute to the selection of a DNA extraction method, and methods must often be adapted to the target organisms and conditions present (Kuhn *et al.*, 2017). While current literature is overflowing with DNA extraction methods either for the purpose of extracting eDNA or *B. dendrobatidis* DNA, none of these assays have been used to detect fungal environmental DNA in South African waters. This study evaluated four DNA extraction methods consisting of two commercial DNA extraction kit methods, and two crude extraction methods, specifically for the purpose of extracting environmental fungal DNA. The benefits and limitations of the different methods were analysed to aid in the selection of two methods for the subsequent filter tests. Crucial considerations and laboratory protocols were considered as well as the importance of high quality and quantity DNA for diagnostic purposes.

2.1.1 Commercial kits vs crude extraction methods

Multiple considerations exist for DNA extraction protocols that are often determined by the target organism. Fungi possess thick cell walls that present challenges to DNA extractions, due to incomplete lysis of the cells (Fredricks *et al.*, 2005). To effectively extract DNA, the cell walls as well as the membranes around the nucleus, need to be disrupted or lysed either chemically, electrically, mechanically, or acoustically (So *et al.*, 2014). Fungal cells are also known for possessing high levels of polysaccharides that have to be removed along with other proteins, lipids, carbohydrates, and organic structures, in order to purify the DNA samples (Kuhn *et al.*, 2017). The purification of DNA is extremely important for downstream application, and the presence of the mentioned components may result in an inhibitory effect or reduce the efficiency of the qPCR assay (Schrader *et al.*, 2012), leading to false negatives or the underestimation of infection load.

Although DNA extraction kits are one of the most applied methods, it is much more cost-intensive compared to crude extraction methods; especially when large quantities of samples have to be analysed (Kuhn *et al.*, 2017). Commercial extraction kit methods are also generalised and can rarely be adapted according to the target organism, but are very reliable due to their consistency and easy application (Kuhn *et al.*, 2017). Both crude DNA extraction methods and commercial kits have previously been used for *B. dendrobatidis* and eDNA studies. Kit DNA extraction methods are known for delivering high quality DNA, but some organic compounds are not always effectively removed (Barbier *et al.*, 2019). In this instance, crude extraction methods are often favoured because they are a cost-effective alternative to commercial kits, and can easily be modified according to the target organisms or environments being sampled (Piggott, 2016, Barbier *et al.*, 2019). It should, however, be noted that some of these methods tend to be more time-consuming and often use hazardous reagents, such as phenol or chloroform (Piggott, 2016, Barbier *et al.*, 2019).

Methods such as the cetyltrimethyl ammonium bromide (CTAB) method and phenol-chloroform-isoamyl methods are some of the most applied crude extraction methods in eDNA studies, as well as for fungi

(Zhang *et al.*, 2010, Ferencova *et al.*, 2017, Tsuji *et al.*, 2019). In previous eDNA studies crude extraction methods, such as CTAB, have occasionally shown better performance than kit methods, such as the Qiagen DNeasy Blood and Tissue Kit, and the PowerWater Kit (Tsuji *et al.*, 2019). The CTAB method has also previously been applied to *B. dendrobatidis* as well as eDNA studies, and delivered positive results (Annis *et al.*, 2004). Multiple variations of this method exist, and the time required to conduct the extractions also vary greatly, from a few hours to more than a day (Tripathy *et al.*, 2017). CTAB is used to disrupt the cell membranes; but other disruption methods such as glass beads, thermal exposure, or chemical disruption methods can be applied along with the CTAB for the complete lysis of cells (Zhang *et al.*, 2010). Chemicals such as chloroform or phenols are then applied to purify the samples (Zhang *et al.*, 2010, Tripathy *et al.*, 2017). Some CTAB methods have been altered to use proteinase K to first lyse the cell walls, and then apply chemicals such as phenol or chloroform to remove the protein components (Chi *et al.*, 2009, Barnes *et al.*, 2020). Phenols break down cellular components that would contaminate the DNA samples; and when combined with water can be spun down due to the differences in density of the mediums, leaving the DNA in suspense (Tripathy *et al.*, 2017). Chloroform is used to dissolve the proteins and lipids and separates them from the DNA; and is also spun down, leaving the DNA in the upper phase of the solution (Tripathy *et al.*, 2017). This step is normally followed by applying isopropanol and ethanol, used to precipitate the samples (Zhang *et al.*, 2010). Another well-known crude DNA extraction method for *B. dendrobatidis* is the PrepMan Ultra method. This was first used by Boyle *et al*. (2004) and applies 40 µL of PrepMan Ultra along with Zirconium beads to mechanically disrupt the *B. dendrobatidis* cells. This method has been applied in many *B. dendrobatidis* studies due to its cost-effectiveness and simplicity (Hyatt *et al.*, 2007, Bletz *et al.*, 2015, Talley *et al.*, 2015). However, when compared to certain commercial extraction kit methods, such as the Qiagen Blood and Tissue Kit, PrepMan Ultra tends to have a greater inhibitory effect despite the dilution of the product; which negatively affects the qPCR results (Bletz *et al.*, 2015). This method has performed similarly to some kits when compared at higher DNA concentrations, however the lower detection rates of the method are more limited due to being less effective at removing inhibitors. Although PrepMan Ultra is a very commonly applied method for *B. dendrobatidis,* it may not be as effective for eDNA samples, due to this decreased sensitivity at lower concentrations of DNA, a condition that is typical for environmental samples.

The Heat Lysis method is a crude extraction method that has previously been used for other fungal pathogens, and is considered very simple and cost-effective, as it only applies Chelax-100 beads and nuclease-free water (Greeff *et al.*, 2012). It is also commonly used in applications with low copy numbers of DNA; for example, blood samples and fingerprints in forensic investigations, small blood samples in virology tests, and cultures in microbiology-based studies on fungi and spores (Turan *et al.*, 2015, Ferencova *et al.*, 2017). Samples are homogenised in nuclease-free water and then transferred to a tube containing the Chelax-100 beads which are negatively charged (Greeff *et al.*, 2012). However, different methods of applying Chelax-100 resins are also commonly used, and methods for extraction vary from lab to lab based on the target organism (Turan *et al.*, 2015, Tripathy *et al.*, 2017). DNA is released from the cells through a boiling process in the presence of Chelax-100 (Greeff *et al.*, 2012, Turan *et al.*, 2015). These beads have metal chelating properties which prevent the degradation of the DNA at boiling temperature, while also binding to metal ions that serve as a catalyst to DNA degradation (Greeff *et al.*, 2012, Turan *et al.*, 2015). This method is known to yield high concentrations of DNA due to minimal transfer steps (Panda *et al*., 2019; Walsh *et al*., 2013); an assay that shows great potential for eDNA studies.

A wide variety of commercial DNA extraction kits have previously been tested to extract DNA from *B. dendrobatidis* as well as aquatic eDNA samples (Bletz *et al.*, 2015). Kits tend to be the most widely utilised DNA extraction method in eDNA studies, due to their simplicity and effectiveness in removing inhibitors (Tsuji *et al.*, 2019). Some of the most used commercial kit methods for aquatic environments are the DNeasy Blood and Tissue Kit, DNeasy PowerSoil Kit, the Quick-gDNA spin-column Kit, MoBio Qiagen PowerWater Kit, MoBio PowerWater Kit and QIAamp Micro Extraction Kit (Rees *et al.*, 2014,

Eichmiller *et al.*, 2016, Tsuji *et al.*, 2019). While the literature reports on a wide variety of extraction kits being used, there are very few reports on the methods used to determine the most effective and optimal kit. It is therefore questionable whether the kits being applied are indeed the most effective. Some kits may provide better DNA yields, whereas others may provide higher quality DNA and remove inhibitors more effectively. For the case of *B. dendrobatidis* one of the most applied kit methods is the Qiagen Blood and Tissue Kit (Bletz *et al.*, 2015). However, more recent studies reported on more effective kits; namely Zymo Research Bacterial and Fungal Kit, and the DNeasy PowerSoil Kit.

2.2 METHODS

Three different culture matrixes were prepared to test the effectiveness of the DNA extraction methods under various conditions. In order to understand potential limitations of the DNA extraction methods, as well as to test for possible downstream qPCR inhibitors coming from the environment; one of the matrixes represented natural environmental conditions. Another matrix provided insight to the degradation of dead *B. dendrobatidis* cells over time, and whether this rate is constant between methods. Both matrixes were compared to a sterile matrix.

Four DNA extraction methods were compared: two crude extraction methods and two kit methods.

2.2.1 Preparing culture matrixes

Cultures were prepared and diluted using 1% tryptone to a concentration of 100 000 zoospores/ mL for all the samples. A final volume of 1 mL was prepared with the selected concentration. Samples were prepared in triplicate for each matrix and DNA extraction method. The three matrixes that were tested were: the sterile, which served as the standard; the non-sterile, which represented environmental conditions; and the heat-treated matrix, which provided insight into dead *B. dendrobatidis* cells over time.

2.2.1.1 Sterile culture

The sterile culture consisted of a known concentration of zoospores, suspended in 1% tryptone broth. All samples were diluted to 100 000 zoospores/ mL. The values from this culture were used as the set standard to which the other matrixes were compared.

2.2.1.2 Non-sterile culture

The non-sterile cultures were prepared through spiking borehole water collected from the NWU botanical gardens with a known concentration of zoospores. Other micro-organisms and possible inhibitors were therefore present and represented an environmental sample. When sampling from the environment, qPCR inhibitors and contaminants may be present that could potentially decrease the sensitivity of the protocol (Lance & Guan n.d., Albers *et al.*, 2013, Stoeckle *et al.*, 2018). This matrix tested the effectiveness of each DNA extraction method to remove possible inhibitory agents and contaminants.

2.2.1.3 Heat-treated culture

Unlike most chytrids, *B. dendrobatidis* does not possess a resting spore phase (Berger & Hyatt, 1999). The resting spore phase is a mechanism that allows organisms, when presented with unfavourable conditions, to enter a resting phase until more optimal conditions allow it to resume with life unaffected

(Berger & Hyatt, 1999). Therefore *B. dendrobatidis* spores may not survive unfavourable conditions. This can significantly affect the amount of eDNA available in the environment. Understanding the time frame of how long DNA takes to degrade after *B. dendrobatidis* cells have died, is important for two reasons. Firstly, if degradation of the cells occurs too quickly, the amount of DNA available for analysis will be reduced; thus, adding to the importance of the insurance of filtering the water in the field, and not transporting it to a laboratory prior to filtration. Secondly, it will assist with the interpretation of the current environmental status relative to the eDNA results. In other words, it will help to determine whether the outbreak is current or whether it is over, while still being able to detect the DNA.

Batrachochytrium dendrobatidis zoospores are heat sensitive and cannot survive at temperatures above 28ºC (Piotrowski *et al.*, 2004). However, they do not immediately degrade after exposure. Zoospores require specific exposure times at different temperatures before they die: at 37ºC spores will perish after 4 hours; at 47ºC it would take 30 minutes; and at 60ºC they only need to be exposed for 5 minutes (Johnson & Speare, 2003). To prepare this matrix, the culture was heat-treated by placing the Eppendorf containing the culture, in a water bath for 30 minutes at 47°C. Following the heat treatment, the cultures were incubated at 20°C for a week prior to extraction, to allow cell degradation to occur.

2.2.2 DNA extraction methods

Two commercial DNA extraction kit methods and two crude extraction methods were tested. The two kit extractions were the Zymo Research Bacterial and Fungal Kit, and the DNeasy PowerSoil Kit. The two crude extractions were a Heat Lysis (Greeff *et al.*, 2012), and a CTAB (Cetyltrimethyl ammonium bromide) extraction. These methods were selected based on their previous success with fungal pathogens or eDNA studies.

2.2.2.1 DNeasy PowerSoil Kit

The DNeasy PowerSoil Kit (Qiagen Catalogue no: QIA/12888-100) has previously been applied to an eDNA study for *B. dendrobatidis* detection in Louisiana, USA; and has shown the delivery of high-quality DNA with accurate results. In 2019 Tsuji and colleagues summarised available published data regarding eDNA assays, and pointed out that in water samples where high levels of inhibitors were present, the DNeasy PowerSoil Kit has repeatedly proved to effectively remove all inhibitors (Tsuji *et al.*, 2019).

Cultures were centrifuged at 10 000 x g for 5 minutes, to remove part of the supernatant. The pellet was resuspended in the remaining supernatant and transferred to a tube containing beads. Samples were placed in the bead beater for 5 minutes at full speed. The manufacturer's instructions were followed for the rest of the protocol.

2.2.2.2 Zymo Research Bacterial and Fungal Kit

Cultures were prepared in the same way as for the DNeasy PowerSoil Kit, and samples were placed in the bead beater for 5 minutes at full speed. One step in the protocol had to be changed due to the "Zymo-Spin IV Filters" being absent in the kit. For this step, the samples were meant to be centrifuged to enable them to filter through the filters, to aid in removing excess cellular debris. This step was replaced by centrifuging the solution at 8 000 x g for 1 minute and transferring the supernatant to a new tube. The rest of the protocol was followed as described by the manufacturer's instructions.

2.2.2.3 Heat Lysis

The Heat Lysis method is a quick and cost-effective protocol that does not require any hazardous chemicals. The method is derived from the study by Greeff *et al.* (2012) and was slightly modified for the target organism. This method has not previously been used in *B. dendrobatidis* studies but was selected due to its success on other fungal pathogens. Fungal cells are known to have thicker cell walls that complicate DNA extractions (Greeff *et al.*, 2012). This method has proven to be effective in this regard and disrupts the cell walls using a hand-held homogeniser. It was the least costly method, requiring only Chelax-100 beads and nuclease-free water.

Samples were centrifuged at 10 000 x g for 5 minutes and the supernatant was removed, leaving 50 µL and a pellet (not visible to the naked eye) behind, after which 250 µL nuclease-free water was added. Samples were homogenised with a custom-made handheld homogenising tip attached to a Dremel tool for 1 minute 30 seconds. The tip of the homogeniser was cleaned between samples, using 10% bleach, followed by 70% ethanol and then ddH_2O: each for a 30sec cycle between every sample. Homogenised samples were transferred to an Eppendorf tube containing 0.04 ± 0.005 g Chelax-100 beads. The samples were briefly vortexed and incubated at 56ºC for 20 minutes. Following incubation, the tubes were vortexed briefly and incubated again at 95ºC for 30 minutes. After this incubation, the tubes were transferred to ice and left for 5 min before being centrifuged at 17 000 x g for 5 minutes at 4ºC. The DNA was in suspense and 150 µL of the supernatant was transferred to a new tube and stored at -80ºC for analysis.

2.2.2.4 CTAB

The CTAB method makes use of CTAB and proteinase K in a thermal lysis process to extract the DNA; and then uses chloroform and isopropanol for the purification of DNA. CTAB methods in general have shown to effectively extract DNA from plant and fungal cells and is known to be a cost-effective method that removes inhibitory agents (Zhang *et al.*, 2010).

Samples were prepared in the same way as the Heat Lysis method, up to when the homogenising step was completed. Following homogenisation, the samples were placed in a freezer at -50°C for 30 minutes, followed by 15 minutes incubation at 65°C in a water bath. After the incubation steps, 3 µL proteinase K was added, and the samples were vortexed and incubated again at 65°C for 60 minutes. The first wash step followed, by adding 300 µL chloroform and gently mixing it with the sample by pipetting up and down a few times. This was followed by centrifugation for 15 minutes at 3 800 x g, and the upper 260 µL was transferred to a new tube. A second wash with chloroform was performed using 87 µL chloroform, and the samples were vortexed and followed by centrifugation for 5 minutes at 12 000 x g. The upper 200 µL was transferred to a new tube and 133 µL ice-cold isopropanol was added. Samples were incubated for 15 minutes at 4°C before being centrifuged at 16 000 x g for 5 minutes. The supernatant was removed and 100 µL of ice-cold 70% ethanol was added and vortexed. The supernatant-ethanol suspension was centrifuged for 5 minutes at 16 000 x g. After centrifugation, the supernatant was removed leaving a pellet. Samples were left to air dry at 65ºC in a bio-flow cabinet with open caps. Pellets were resuspended in 100 µL TE buffer and stored at -80°C.

2.2.2.5 Evaluation of methods

Nano-drop spectrophotometry was applied along with TaqMan Probe qPCR to determine the quality, quantity, Ct-values, and level of variance within; as well as between, the different matrixes. Lower Ct-values is an indication of overall better quality and higher quantity DNA. The quality of the Nano-drop

spectrophotometer was measured using the A260/A280 ratio. A value between 1.8 and 2.1 is considered good quality DNA. Values beneath 1.8 may contain protein contamination, which could possibly cause inhibition in the qPCR phase and values above 2.1 may contain RNA contamination (Koetsier & Cantor, 2019). The quantity of the samples was measured in ng/µL.

Extra care was taken to ensure no contamination. The reagents, quantities, methods, and *B. dendrobatidis* strain remained the same throughout the entire molecular phase of this project. Previous studies conducted by Hyatt *et al.* (2007) and Blooi *et al.* (2013) has shown that the qPCR primers and assay, also used in this study, have a very high level of reproducibility between runs, and even across machines. In addition, the efficiency of the primers was confirmed in this study. (See Chapter 4).

2.2.2.6 Statistical analyses

The statistical analysis was done using GraphPad Prism v8.0.2 to determine mean differences and statistical significance. A series of one-way ANOVAs were used to determine the statistical significance for qPCR Ct-values. The sterile and non-sterile matrix were analysed separately and then an overall analysis was run for both variables. The sample averages were determined from the qPCR replicates for each analysis. A Bonferroni multiple comparison test was performed post hoc to determine the significance of each individual method in comparison to the other methods within each matrix, as well as overall. The Bonferroni multiple comparison test has previously been applied in other studies for the statistical comparison between Ct-values of DNA extraction methods (Auricchio *et al.*, 2013, Psifidi *et al.*, 2015). The data was considered statistically significant when the p-value was less than 0.05.

The matrixes within each DNA extraction method were compared to determine whether any significant differences could be observed in the DNA extraction methods, and if losses in DNA occurred over time between the sterile and non-sterile matrix. This analysis was done using a Two-way ANOVA, with a Bonferroni post hoc multiple comparison test. The data was considered statistically significant when the p-value was less than 0.05.

The mean, standard deviations (SD) and coefficient of variability percentage (CV%) were determined using Excel 2016 for each matrix and the methods overall. The CV% was determined through the equation:

CV% = (SD/Average) *100

2.3 RESULTS

2.3.1 Quantity (ng/µL)

Two-way ANOVA results showed no significant difference ($p = 0.2332$) between matrixes or methods. When comparing the quantity results between the sterile matrix and heat-treated matrix, the Dneasy PowerSoil Kit and Heat Lysis methods yielded an increase in quantity while the Zymo Research Bacterial Fungal Kit and CTAB methods had decreased quantities (Figure 2-1; Table 2-1; Table 2-2). The only significant difference detected was in the Zymo Research Bacterial and Fungal Kit method ($p = 0.0330$) (Table 2-2). All the other readings in the post hoc Bonferroni multiple comparison test did not yield any significant difference in the DNA yield between the sterile, non-sterile and heat-treated matrixes for any of the methods (Table 2-2).

2.3.2 Quality (A260/A280)

The quality readings differed significantly in the overall Two-way ANOVA analysis ($p = 0.0007$). When reviewing the kit methods, no significant differences could be detected between any of the matrixes in the post hoc Bonferroni multiple comparison test (Table 2-3). In contrast, significant differences were found for both the crude extraction methods between the different matrixes. In the Heat Lysis method, a significant difference could be observed between the non-sterile and heat-treated qualities ($p = 0.0090$) (Table 2-3). This is due to the very high readings presented by the heat-treated matrix which had an average above 2.1, indicating possible RNA contamination (Table 2-1). Significant differences were observed between the sterile and heat-treated matrix ($p = 0.0065$) as well as non-sterile and heat-treated matrix ($p = 0.0009$) of the CTAB method (Table 2-3). This may have been due to the odd readings presented by the Nano-drop spectrophotometer that indicated a drop in quality and quantity for the heat-treated matrix.

2.3.3 Ct-values

All four DNA extraction methods experienced a significant increase in Ct-values, thus a decline in the DNA present, when comparing the sterile matrix to the heat-treated matrix (Figure 2-1). Thus, a significant loss in DNA occurred over time when no preservation medium was applied. Although all the methods experienced an increase in their Ct-values compared to the sterile matrix, this increased value demonstrated variation between the methods, and indicated that the degradation rates may have differed. The level of degradation varied from 1.737-4.090 Ct-values (Table 2-4).

When comparing the sterile matrix to the non-sterile matrix Ct-values for each individual method, differences could be observed between the kit methods and crude extraction methods. A significant increase in Ct-values was observed in the non-sterile matrix compared to the sterile matrix for both the crude extraction methods (Table 2-4); whereas the kit methods did not experience a significant change in the non-sterile matrix compared to the sterile matrix (Table 2-4). This possibly indicates that the kit methods are more effective at removing the inhibitors present in non-sterile samples, compared to the crude extraction methods. It should, however, be noted that the Ct-values for the non-sterile matrix, as previously indicated, does not differ significantly between the crude and kit extraction methods and all four methods performed similarly in this matrix.
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Matrix comparison results for DNA extraction methods

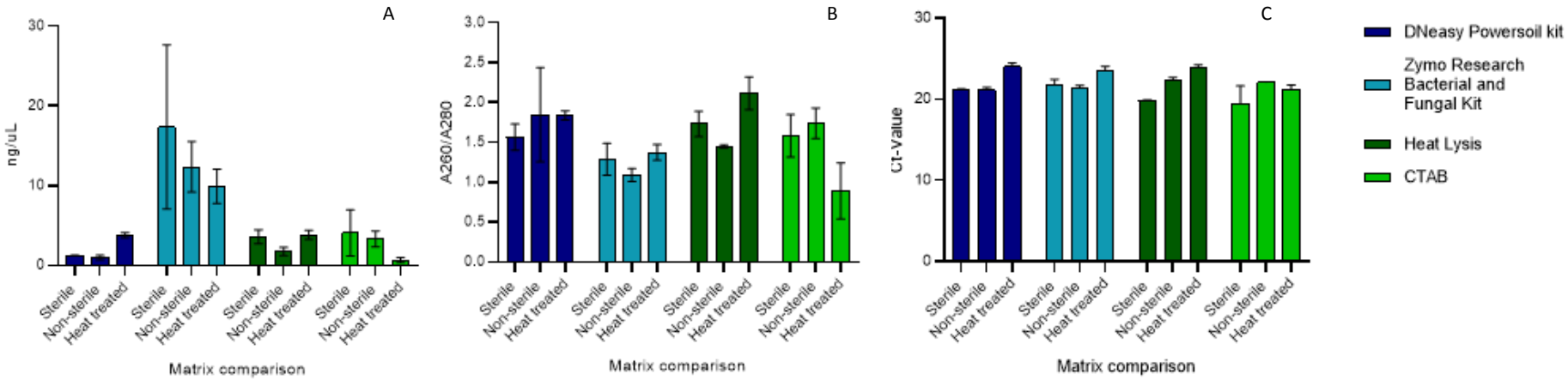

FIGURE 2-1: Matrix quantity (ng/µL), quality (A260/A280) and Ct-value results for all DNA extraction methods, (A) Matrix quantity result comparison and sample range for all DNA extraction methods, (B) Matrix quality result comparison and sample range for all DNA extraction methods, (C) Matrix Ct-value result comparison and sample range for all DNA extraction methods.

TABLE 2-1: Heat-treated matrix quantity (ng/µL), quality (A260/A280) and Ct-value means, standard deviations (SD) and coefficient of variability (CV%) for the DNA extraction methods.

DNA extraction method	Mean quantity ± SD (CV%)	Mean quality ± SD (CV%)	Mean Ct-value ± SD (CV%)
Dneasy PowerSoil Kit	3.77 ± 0.32 (8.41)	1.84 ± 0.06 (3.19)	24.00 ± 0.43 (1.78)
Zymo Research Bacterial and Fungal Kit	9.90 ± 2.15 (21.71)	1.37 ± 0.10 (7.29)	23.57 ± 0.45 (1.92)
Heat-lysis method	3.83 ± 0.57 (14.83)	2.12 ± 0.20 (9.65)	23.90 ± 0.35 (1.47)
CTAB method	0.64 ± 0.31 (48.07)	0.89 ± 0.35 (39.36)	21.16 ± 0.58 (2.75)

TABLE 2-2: Sterile, non-sterile and heat-treated matrix comparison for Quantity values (ng/µL) using a two-way ANOVA Bonferroni post hoc multiple comparisons test between matrix types (p-values in red are significant).

DNeasy PowerSoil Kit		
Method comparison	**Mean difference**	**P-value**
Sterile vs Non-sterile	0.2467	>0.9999
Sterile vs Heat-treated	-2.543	>0.9999
Non-sterile vs Heat-treated	-2.790	0.9386
Zymo Research Bacterial Fungal Kit		
Method comparison	**Mean difference**	**P-value**
Sterile vs Non-sterile	4.993	0.2322
Sterile vs Heat-treated	7.457	0.0330
Non-sterile vs Heat-treated	2.463	>0.9999
Heat Lysis		
Method comparison	**Mean difference**	**P-value**
Sterile vs Non-sterile	1.847	>0.9999
Sterile vs Heat-treated	-0.2433	>0.9999
Non-sterile vs Heat-treated	-2.090	>0.9999
CTAB		
Method comparison	**Mean difference**	**P-value**
Sterile vs Non-sterile	0.7467	>0.9999
Sterile vs Heat-treated	3.413	0.6581
Non-sterile vs Heat-treated	2.667	>0.9999

TABLE 2-3: Sterile, non-sterile and heat-treated matrix comparison for Quality (A260/A280) using a two-way ANOVA Bonferroni post hoc multiple comparisons test between matrix types (p-values in red are significant).

DNeasy PowerSoil Kit		
Method comparison	**Mean difference**	**P-value**
Sterile vs Non-sterile	-0.003667	>0.9999
Sterile vs Heat-treated	-2.875	0.0003
Non-sterile vs Heat-treated	-2.871	0.0003
Zymo Research Bacterial Fungal Kit		
Method comparison	**Mean difference**	**P-value**
Sterile vs Non-sterile	0.3760	>0.9999
Sterile vs Heat-treated	-1.796	0.0215
Non-sterile vs Heat-treated	-2.172	0.0048
Heat Lysis		
Method comparison	**Mean difference**	**P-value**
Sterile vs Non-sterile	-2.554	0.0010
Sterile vs Heat-treated	-4.090	<0.0001
Non-sterile vs Heat-treated	-1.536	0.0571
CTAB		
Method comparison	**Mean difference**	**P-value**
Sterile vs Non-sterile	-2.670	0.0006
Sterile vs Heat-treated	-1.737	0.0269
Non-sterile vs Heat-treated	0.9328	0.4196

TABLE 2-4: Sterile, non-sterile and heat-treated matrix comparison for Ct-values using a two-way ANOVA Bonferroni post hoc multiple comparisons test between matrix types (p-values in red are significant).

DNeasy PowerSoil Kit		
Method comparison	**Mean difference**	**P-value**
Sterile vs Non-sterile	-0.003667	>0.9999
Sterile vs Heat-treated	-2.875	0.0003
Non-sterile vs Heat-treated	-2.871	0.0003
Zymo Research Bacterial Fungal Kit		
Method comparison	**Mean difference**	**P-value**
Sterile vs Non-sterile	0.3760	>0.9999
Sterile vs Heat-treated	-1.796	0.0215
Non-sterile vs Heat-treated	-2.172	0.0048
Heat Lysis		
Method comparison	**Mean difference**	**P-value**
Sterile vs Non-sterile	-2.554	0.0010
Sterile vs Heat-treated	-4.090	<0.0001
Non-sterile vs Heat-treated	-1.536	0.0571
CTAB		
Method comparison	**Mean difference**	**P-value**
Sterile vs Non-sterile	-2.670	0.0006
Sterile vs Heat-treated	-1.737	0.0269
Non-sterile vs Heat-treated	0.9328	0.4196

2.4 DISCUSSION

Nano-drop spectrophotometry readings revealed high variations, possibly due to low DNA concentrations. Sample concentrations are considered low when they are less than 50ng/µL (Koetsier & Cantor, 2019), and all the samples for all the methods in this chapter yielded concentrations below this value. This led to greater variations in the data sets that provide the larger CV% values for both the quantity and the quality readings. Sample quality is often more important than DNA quantity, because a qPCR assay requires a small amount of high-quality DNA to successfully amplify the target DNA (Walsh *et al.*, 2013).

The sterile matrix was used as the standard to which the other two matrixes were measured. All samples across all three matrixes started with the same concentration of zoospores. In addition, four different extraction methods were tested on all three matrixes. This was useful to determine whether the methods were able to effectively remove possible qPCR inhibitors from an environmental matrix.

Overall, the sterile samples that were extracted using the two crude extractions both resulted in lower Ct-values, compared to the Ct-values of samples that were extracted using the kits, while the non-sterile samples that were extracted using the crude extractions showed significantly higher Ct values. Lower Ct-values is an indication of higher quantity DNA and/or higher quality DNA. Commercial kit methods are known for yielding lower quantities of DNA due to the multiple cleaning steps, but often yield high quality of DNA (Barbier *et al.*, 2019). The benefits of this can be seen when analysing the non-sterile matrix. The non-sterile matrix contained inhibitors that would typically be associated with an environmental sample. Both commercial DNA extraction kit methods performed better than the crude extraction methods with regards to Ct-values, potentially indicating more effective removal of inhibitors.

Despite the low-quality readings for the samples extracted with the Zymo Research Bacterial Fungal Kit, these samples presented better Ct-values than those samples extracted with crude methods.

The main purpose of the heat-treated matrix was to determine whether significant decreases in DNA can be observed for the methods over the period of a week if no preservation medium is applied. According to previous literature, dead cells can persist for longer than two weeks if presented with the ideal environmental conditions (Schmidt *et al.*, 2013). These degradation rates may, however, differ between species and vary based on the environmental conditions present (Andruszkiewicz Allan *et al.*, 2021). All four DNA extraction methods experienced a significant increase in Ct-values over the period of a week. This is an indication that significant quantities of DNA can be lost over a period of a week, thus, the use of preservation mediums in filter material had to be tested.

CHAPTER 3: SAMPLE COLLECTION AND PRESERVATION

3.1 INTRODUCTION

Filtration has previously been applied in multiple *B. dendrobatidis* and amphibian eDNA studies (Brannelly *et al.*, 2020, Peixoto *et al.*, 2021). In the filtration method, a filter medium is used through which water flows, then DNA gets trapped and extracted from the filter media (Hinlo *et al.*, 2017). Filtration is one of the most applied methods for eDNA studies due to its capability to filter larger volumes, and often yields more DNA than precipitation (Piggott, 2016, Tsuji *et al.*, 2019). There are, however, several factors to consider when using filtration, such as the type of filter material, pore sizes, and filter volumes; and therefore, methods must be adjusted based on the target organism.

When samples cannot be processed immediately following collection, a preservation medium for the filters is required. Due to the rapid degradation rates often observed in DNA, it is recommended to filter the samples directly from the source and use a preservation medium, rather than transporting water samples to be analysed at a later stage (Kumar *et al.*, 2020). Some of the most used reagents for preservation include ethanol fixation, lysis buffers, freezing, and silica gel/beads.

Silica gel beads have been applied in various previous eDNA studies for the preservation of filter media (Majaneva *et al.*, 2018, Hansen *et al.*, 2019). This preservation method has shown to successfully preserve eDNA with minimal loss over a month, with consistent results. To prevent any significant decreases in DNA for a longer period the samples could be stored at -20°C.

Chilling or freezing samples is often used for eDNA preservation of filter materials and water samples (Kumar *et al.*, 2020). Freezing samples will reduce the rate of degradation but does not prevent it altogether. In addition, freezing and thawing of samples will have a negative impact on the eDNA. The sampling sites selected for this project occur within remote regions that often do not have electricity available, thus freezing was not an option.

Ethanol, ranging from 70% to 95%, has proven to be sufficient for eDNA preservation in multiple studies, and has even previously been used for the preservation of *B. dendrobatidis* swabs and amphibian tissue samples (Rees *et al.*, 2014, Talley *et al.*, 2015, Piggott, 2016). This method has also shown over time to preserve DNA better than freezing samples and can limit DNA degradation for up to 172 days. This medium is considered both a cost and time effective method of eDNA preservation (Hinlo *et al.*, 2017).

The Longmire's Lysis Buffer has been used in many studies with remarkable success (Renshaw *et al.*, 2015, Kumar *et al.*, 2020, Mauvisseau *et al.*, 2021). Originally this buffer was developed for the long-term preservation of tissue samples, but more recently this same method was applied for eDNA application (Williams *et al.*, 2016). This buffer coupled with cellulose nitrate filters and the DNeasy Blood and tissue Kit has shown to be one of the most utilised methods for the successful capture of eDNA (Kumar *et al.*, 2020). The Lysis Buffer has also shown that eDNA remains intact for up to 150 days at room temperature, with reliable detection results and lower variation when compared to freezing and other buffers, such as Sarkosyls buffer (Renshaw *et al.*, 2015, Wegleitner *et al.*, 2015, Mauvisseau *et al.*, 2021).

3.2 WATER COLLECTION

The three selected filtration methods were the drill filter, Continuous Low-level Aquatic Monitoring (C.L.A.M.), and the syphon pump method. These methods were mainly tested in the field to determine

time investment, optimum water volume before filter clogging occurred, and the practicality of the methods for application in remote regions. The benefits and limitations of the methods were compared to determine appropriateness for future field sampling and subsequent testing and optimising the sampling assay in the laboratory. Five samples were taken using each of the methods aiming to filter approximately 1 L of water per sample, or until clogging of the filter occurred.

3.2.1.1 Drill filter

This method was selected based on its previous eDNA applications for aquatic pathogen detection (Hansen *et al.*, 2019). This filtration method was able to successfully detect the host specimen in every sample and occasionally the selected pathogen. The lack of detection of the pathogen was, however, not attributed to the filtration method, but rather to the prevalence of the pathogen within the environment that was below the detection limit of the assay (Hansen *et al.*, 2019). The drill filter method uses a drill to power a pump, creating a vacuum that draws water through the filter media. The following components were used to construct the filter: a filter cup (Biotechnology Hub Africa. Cat# 145-2045), tubing, a drill powered pump (Gardena, Electric Drill Pump, no 1490-20),and a power drill (Figure 3-1). Filter cups were changed with every sample taken. It is considered a cost-effective alternative to commercial filtration devices and could be applied directly in the field.

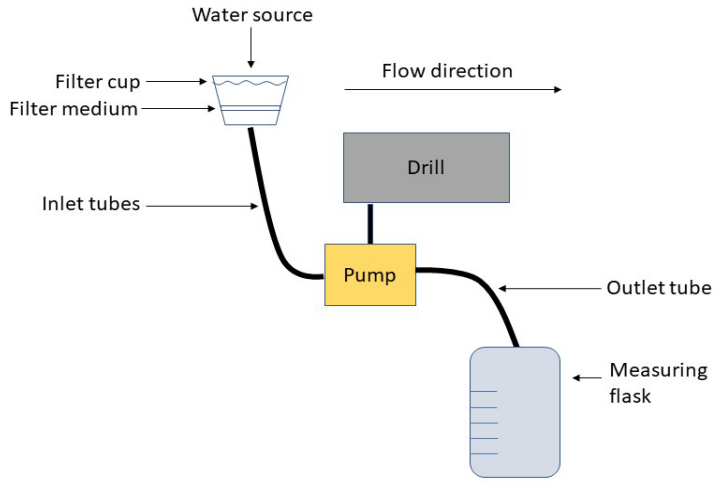

FIGURE 3-1: Diagram of the drill filter pump

Our first trial method used a drill pump to create a suction. We found this method to be the least consistent of the three methods. Drill speed is hard to control, and rather tiring to operate when multiple samples require processing. We trialled two drill types – electrical and battery powered. Surprisingly, both drills failed to create a consistent vacuum that allows water to filter at a “drip”-rate. The electrical drill quickly overheated, due to a lack of cooling air over the motor, brought on by the low drilling speed. On the other hand, the battery-operated drill did not overheat, but ran out of power after filtering approximately 4 L of water. The setup for the drills is rather cumbersome with clamps, 20 mm tubing, buckets and suspension stands. On the plus side it uses filter cups that can readily be purchased from scientific supply companies. Only the battery powered drill can be used in the field, but away from the water source on a stable surface.

3.2.1.2 Continuous low-level aquatic monitoring (C.L.A.M.)

The C.L.A.M. is a commercial filtration product developed by Aqaulitical. Originally this product was developed to test for organic solutions in the upper phase of water bodies. This method applied a solid phase extraction disk to capture organic particles, which are then recovered from the discs through elution. According to the manufacturers this method can filter up to 100 L of water, but filters water at a low flow rate of 5-80 mL/minute. The filter has a built-in battery that can last up to 36 hours. Due to filter material being encased, it is recommended to freeze the discs to preserve samples.

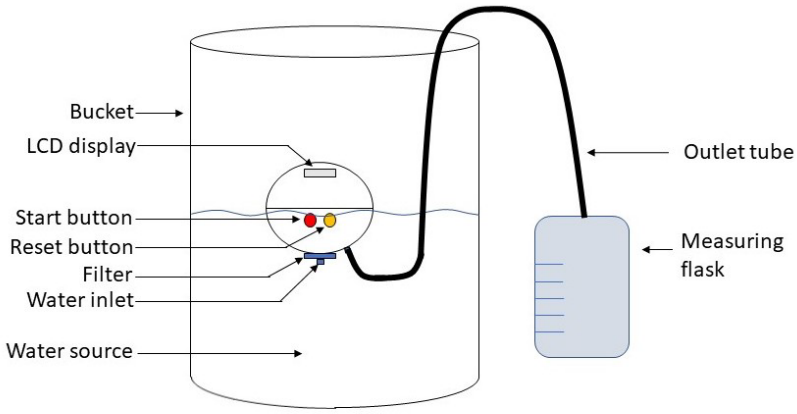

FIGURE 3-2: Diagram of the Continuous low-level aquatic monitoring (C.L.A.M) pump

The second method, The C.L.A.M method delivered a continuous slow filter rate that is highly beneficial for yielding a consistent result. The rechargeable battery proved to be a challenge though, with the charger port taking in water; and with no indication of battery strength one runs the risk of losing power while a sample is in progress. The device floats in the water, but to avoid it taking on water, it is better to suspend it outside of the water source. Charging the battery takes a staggering 12 hours. The device comes with a hefty price tag and uses tailor-made filters, which makes it the least desirable option when resources are a major issue. We predict that the C.L.A.M will operate well in the field, provided that an electrical power supply is available, and ample time for charging can be afforded. Because suspending the device out of the water is not always practical in the field, one must take extra precaution to seal the charger port. We caution that due to the sensitivity of this expensive device, its use in anything other than relatively calm water bodies should be avoided. A practical solution would be to collect water in a sterile bucket for filtering.

3.2.1.3 Syphon pump

The syphon pump method was the most simplistic of the three methods and required no electricity for filtration. The design of this method was inspired by that used by Walker *et al.* (2007), which applied a 50 mL syringe to create a vacuum to filter the water. The syphon pump method consisted of the MAC AFRIC Manual Liquid and Air Siphon pump, two pipes and a filter cup (Figure 3-3). This method was the most cost-effective and very easy to acquire. Using the handheld pump, a vacuum was created

which drew the water through the filter material. This method could filter larger quantities of water more quickly, and with less effort compared to the syringe method. The method was excellent for direct application in the field, due to its light weight and portable size that could easily be carried over great distances to remote regions. It should be noted that this protocol required a lot more manual labour compared to the previous two methods described.

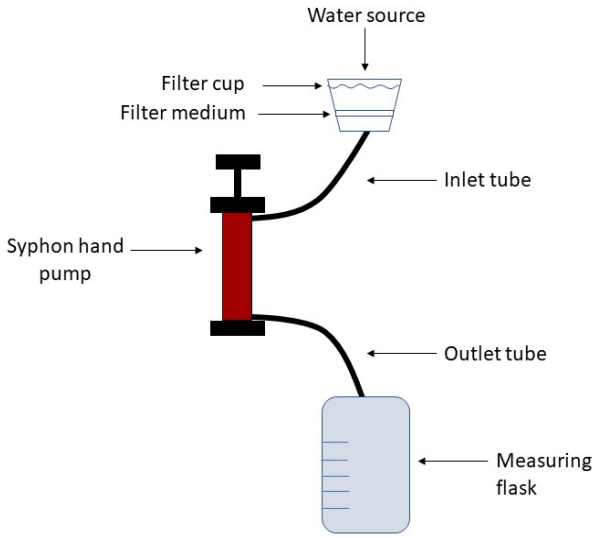

FIGURE 3-3: Diagram of the syphon pump

The syphon pump resulted in very satisfying operating conditions. The basic setup was like that of the drill pump, with the exception that no external power source is required, and it makes use of 10 mm tubing. The fact that the device is manually operated allows for dextrous control over the vacuum, resulting in a consistent filter rate. The device is extremely lightweight, and so it does not result in operator fatigue as with the drills. Not being dependent on electricity implies that the syphon pump can be used anywhere, anytime, and it has the added bonus of being very cheap – it retails at approximately R100 at selected outlets (e.g. Adendorff Machinery Mart). Depending on the length of the tubing, the device can be used to either filter water directly from the source or filter from a bucket collected at the water source. Considering efficacy, reliability, practicality, and pricing; we recommend that the syphon pump be the first method of choice for collecting eDNA samples.

3.3 SAMPLE PRESERVATION

3.3.1 Methods

For this study 2 preservation mediums were tested, each for three different time intervals. The two mediums included 70% Ethanol and Longmire's Lysis Buffer (Longmire & Baker, 1997). The three time intervals tested were <24h, 3d, and 7d (for the purpose of this study when samples were extracted on the same day, immediately after sample preparation and filtering, it was categorised as <24h). Longmire's Lysis Buffer and 70% ethanol were selected as the preservation mediums to test during this study, due to its previous success in eDNA literatures (Renshaw *et al.*, 2015, Talley *et al.*, 2015, Hinlo *et al.*, 2017, Kumar *et al.*, 2020). The Longmire's Lysis Buffer was prepared using the procedure described by Longmire *et al*. (1997). To prepare 1 L of the buffer 200 mL Ethylenediaminetetraacetic Acid (EDTA concentration: 0.5 and pH: 8.0), 50 mL Tris Hydrochloride (Tris-HCl concentration: 2M and pH: 8.0), 2 mL Sodium Chloride (NaCl concentration: 5M) and 25 mL of 20% SDS (Sodium Dodecyl Sulphate) was mixed and added to 975 mL ddH_20, and the solution was autoclaved (Longmire & Baker, 1997).

Samples containing *B. dendrobatidis* cultures were prepared for use during testing. Erlenmeyer flasks were filled with 150 mL of ddH_2O and autoclaved. *B. dendrobatidis* cultures of 1 mL with a concentration of 100 000 zoospores per mL were prepared in triplicate for each experiment. These cultures were used to spike the autoclaved water. One water flask was not spiked, which served as the negative control sample. The negative control was used to determine whether cross-contamination occurred between samples. Water samples were filtered using the pump of choice, syphon pump.

Nalgene Single Use Analytical Funnels with cellulose nitrate filters and a pore size of 0.45 µm (catalogue number: 145-2045) in individual sterile packages were used. The water samples were swirled in the filter cups occasionally during the filtration process to ensure that only a minimum number of zoospores would be lost, due to adhering to the sides of the filter cup.

After filtering the sample, the filter material was cut in half and folded in half using the sterilised tweezers and scissors. The filters were placed in 15 mL falcon tubes containing approximately 1.5 mL of the selected preservation medium. The tubes were sealed with parafilm and left for their selected preservation time (<24h, 3d, or 7d).

Two DNA extraction methods were applied to filters, one kit method and one crude extraction. The filter material processing step followed the same procedure for both the DNA extraction methods, with the only difference being the types of beads and solution applied in each method. For the DNeasy PowerSoil Kit, the PowerBead tube and reagents provided in the kit were used. For the Heat Lysis method (crude extraction), 500 ± 0.01 mg glass beads and nuclease-free water was used. Initially different volumes of nuclease-free water were tested with the glass beads for the Heat Lysis method, to determine the most optimal combination. A supernatant with a volume of 450 µL would need to be available to be transferred to a new tube after the bead beating phase, to allow for a direct comparison to the kit method volumes. Samples with 600 µL and 700 µL of nuclease-free water were prepared for the Heat Lysis method and DNA was extracted from the filter materials. A volume of 650 µL was selected for the protocol because cross-contamination is more likely to occur when using the 700 µL, due to probable spillage.

To process the filter material, filters were cut into smaller pieces and transferred to Eppendorf bead tubes. For the DNeasy PowerSoil Kit, the first reagent as specified in the manufacturer's instructions was added prior to bead beating, and the Heat Lysis method only applied nuclease-free water. Prior to the bead beating step, all samples were vortexed for 5 seconds. Samples were placed in the bead beater at full speed for 15 minutes. After 10 minutes the bead beater was stopped and the samples were vortexed for 5 seconds, in order to redistribute the filter material that had been compressed by the beads into the bottom of the tube; to ensure the full processing of all the filter material pieces. After bead beating, the samples were spun down in a centrifuge at 10 000 x g for 30 seconds, and 450 µL of supernatant was transferred to a new Eppendorf tube. The rest of the DNeasy PowerSoil Kit protocol was followed according to the manufacturer's instructions. For the Heat Lysis method, after the 450 µL supernatant was transferred, the samples were spun down for 5 minutes at 10 000 x g, 400 µL of the supernatant was removed, and 250 µL of nuclease-free water was added to the pellet to serve as an additional cleaning step. Following the last step, the rest of the Heat Lysis method was followed.

According to a study conducted by Walker *et al.* (2007), *B. dendrobatidis* was extracted from filter materials through a similar method as the kit method; but rather than cutting the filter material, the whole filter was placed into the Powerbead tube and processed in a bead beater for 2 minutes. The current study modified this by cutting the filter material, which allowed for better homogenisation of the material because the beads could move effectively between the filter pieces during the bead beating step. Filters

were also placed in the bead beater for 15 minutes rather than 2 minutes to ensure that the maximum amount of DNA was retrieved from the filter materials.
Following extraction, qPCR was used to determine the differences and efficiencies of the extraction methods.

3.3.2 Results

3.3.2.1 Comparison of preservation mediums over different time periods

The Heat Lysis-Lysis Buffer delivered the most consistent results on average over time with the lowest SD (0.69) and CV% (2.71) for the overall results. A Two-way ANOVA Bonferroni multiple comparison test showed that this method demonstrated the least significant changes over time (>0.9999 for all comparisons) (Table 3-1). It should be noted that although the averages of the Ct-values remained more consistent overall and for each day, the greatest increase in CV% could be observed for this method as time increased. For example, on Day 7, this method had the highest SD (1.19) and CV% (4.64) of all the methods, but the average Ct-values from Day 7 varied minimally compared to Day 1. This implies that overall, this method delivers very consistent results over time in terms of its average, but the level of variation between the samples increased as time progressed. Thus, if this method is used, samples should be processed as soon as possible after sampling to reduce variability in the data sets.

The Heat Lysis-Ethanol combination experienced an increase in Ct-values over time, but the increase was very gradual and insignificant. When compared to the Heat Lysis-Lysis Buffer combination, this combination experienced the greatest increase in Ct-values over time and the difference between day 1 and 7 was significant according to a Two-way ANOVA Bonferroni multiple comparison test ($p = 0.0177$). Despite this greater increase in Ct-values, the differences between the Heat Lysis-Lysis Buffer and Heat Lysis-Ethanol combinations were not significant for any of the days observed, nor overall when analysing the one-way and two-way ANOVA results. Thus, both methods preformed very similarly during all the tested experiments. We would, however, recommend rather applying the Ethanol preservation medium for this extraction method; due to its more consistent results and lower loss in DNA over time, despite not performing significantly better than the Lysis Buffer.

The Dneasy Powersoil Kit method combinations followed an irregular trend in Ct-values compared to the Heat Lysis methods over time. Both the Heat Lysis methods demonstrated a gradual increase in the Ct-values over time, whereas the DNeasy PowerSoil Kits demonstrated fluctuating Ct-values. The Dneasy Powersoil-Lysis Buffer combination displayed a significant increase in the Ct-values from <24h to Day 3. The Ct-values for Day 7 were very similar compared to the Day 3 results, but there was no significant difference between the <24h and the Day 7 results. Thus, an increase in Ct-values can be observed within three days when using the Lysis Buffer preservation medium, but this increase may not necessarily always be significant. The rate of DNA loss may possibly decrease after 3 days, which might explain why there is no significant different between Day 3 and Day 7. Due to the greater loss in DNA within the first three days, it would be recommended to process filter material within three days of sampling to ensure the highest quantity results.

No significant differences were observed over time for the DNeasy PowerSoil-Ethanol combination, but this method demonstrated greater fluctuations in Ct-values compared to the DNeasy PowerSoil-Lysis Buffer combination, as seen in the SD value for the overall mean.

Overall, the preservation mediums performed similarly for the Heat Lysis methods, but greater differences could be observed between the DNeasy PowerSoil combinations. For three of the four

tested method combinations, The SD and CV% of the Ct values in the samples increased the longer the samples were preserved in 75% of the tested combinations (extraction method – preservation medium). This is an indication that there is a higher level of variation between samples over time. Thus, quantification of field samples may be less accurate the longer the samples are preserved. Slightly greater increases in the Ct-values were observed for both the methods using the Lysis Buffer preservation medium over time, when compared to the Ethanol preservation medium (Table 3-1). This indicated that the Lysis Buffer may be slightly less effective compared to the 70% ethanol at preserving DNA over time, but both methods still delivered similar results for the Heat Lysis extraction method.

3.3.2.2 Effect of preservation mediums on Ct-values

When comparing the results from <24h to those of the sterile matrix from Chapter 3, a significant inhibition was noticed in 75% of the methods, when comparing Ct-values of combination methods to the Ct-values of extractions performed directly on cultures. The only method combination which did not show a significant loss in DNA in terms of Ct-values was the Dneasy PowerSoil-Lysis Buffer combination ($p = 0.1975$) (Table 3-2). Thus, it can be concluded that this method effectively removes the Lysis Buffer preservation medium and leaves high quality DNA, which differs minimally from a sterile culture DNA extraction. Although ethanol was previously indicated to be more effective at preserving DNA over time, it had the greatest inhibitory effect on the DNeasy PowerSoil Kit and caused the greatest level of variability between days.

TABLE 3-1: Average Ct-value statistical comparison between days within each DNA extraction method and preservation medium combination, using Two-Way ANOVA analysis with Bonferroni multiple comparison test (p-values in red are significant).

DNeasy PowerSoil Kit – Ethanol		
Day comparison	**Mean difference of Ct values**	**P-value**
<24h vs. Day 3	2.203	0.0005
<24h vs. Day 7	1.021	0.1469
Day 3 vs Day 7	-1.183	0.0732
DNeasy PowerSoil Kit – Lysis Buffer		
Day comparison	**Mean difference of Ct values**	**P-value**
<24h vs. Day 3	-1.588	0.0108
<24h vs. Day 7	-1.056	0.1268
Day 3 vs Day 7	0.5322	0.8711
Heat Lysis – Ethanol		
Day comparison	**Mean difference of Ct values**	**P-value**
<24h vs. Day 3	-0.03167	>0.9999
<24h vs. Day 7	-0.1117	>0.9999
Day 3 vs Day 7	-0.0800	>0.9999
Heat Lysis – Lysis Buffer		
Day comparison	**Mean difference of Ct values**	**P-value**
<24h vs. Day 3	-0.8665	0.2733
<24h vs. Day 7	-1.488	0.0177
Day 3 vs Day 7	-0.6212	0.6573

TABLE 3-2: The impact of preservation mediums on the Ct-values for each DNA extraction method and preservation medium combination, using average Ct-values from the sterile matrix of the Heat Lysis method and Dneasy PowerSoil Kit in comparison to the Ct-values from the filter tests (<24h). Analysis done using One-way ANOVA with Bonferroni multiple comparison post hoc test (p-values in red are significant).

DNA extraction method	Preservation medium type	Mean Ct-value ± SD (CV%)	Mean difference to sterile matrix	P-value
Dneasy PowerSoil Kit	Sterile matrix (no preservation)	21.13 ± 0.29 (1.39)	N/A	N/A
	Lysis buffer	21.60 ± 0.05 (0.24%)	0.4688	0.1975
	70% Ethanol	30.99 ± 0.62 (2.00%)	9.860	<0.0001
Heat Lysis method	Sterile matrix (no preservation)	19.81 ± 0.20 (1.03)	N/A	N/A
	Lysis buffer	24.71 ± 0.21 (0.84%)	4.896	<0.0001
	70% Ethanol	25.61 ± 0.16 (0.63%)	5.800	<0.0001

The Lysis Buffer indicated a lower inhibitory effect for both the DNA extraction methods compared to the ethanol for the less than 24-hour period (Table 3-2). Although the Heat Lysis-Lysis Buffer method had lower Ct-values compared to the ethanol during this time, the difference was insignificant. Thus, the DNA was extracted with a similar level of efficiency for both preservation mediums in this method. It should, however, be noted that both preservation mediums in the Heat Lysis method still indicate a significant level of inhibition based on the increase in Ct-values compared to the sterile matrix (Table 3-2). Based on these results, the DNeasy PowerSoil-Lysis Buffer combination was the most successful combination and removed inhibitors with great success. This method was selected as the method of choice for this project and was further tested in the following section to determine the Lowest limit of detection (LOD) and Lowest level of quantification (LOQ) of the assay.

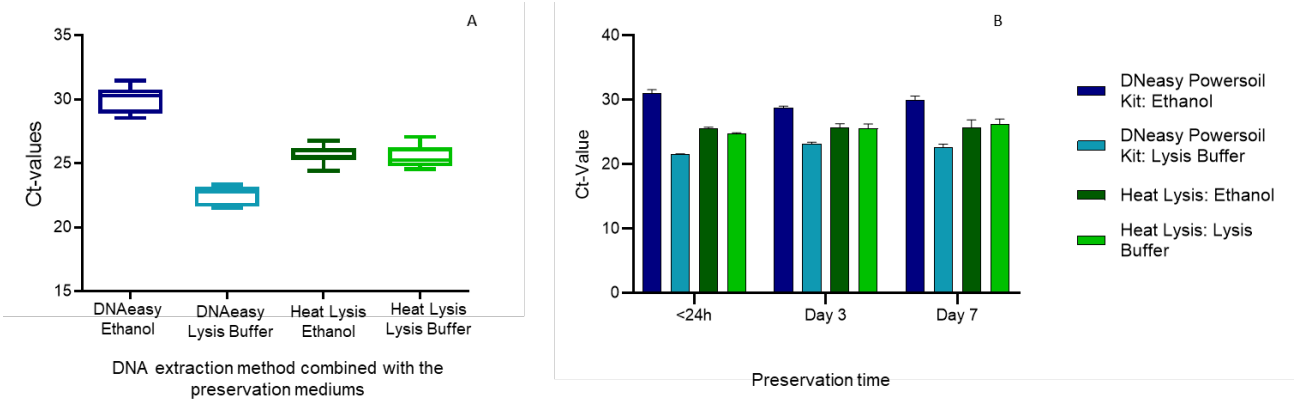

FIGURE 3-4: A) Overall Ct-value for the different combinations of DNA extraction methods and preservation mediums, B) Ct-values over time for different combinations of DNA extraction methods and preservation mediums.

3.3.3 Discussion

The DNeasy PowerSoil Kit with Lysis Buffer preservation medium presented the lowest Ct-values that were statistically significant when compared to the other methods. In the study by Brannelly *et al.* (2020) the Qiagen Powersoil Kit had been selected as the method of choice for eDNA of *B. dendrobatdis*. During the study it was determined that the lowest detection limit of their assay was approximately 100 zoospores. However, in the present study the lowest detection limit was 10 zoospores, based on the

filter dilution series. This may be accounted for by the filter processing steps that were additionally applied in our assay, as well as the application of a buffer for eDNA preservation.

The Heat Lysis-Ethanol combination delivered the most consistent results of all the method combinations over time. Although the ethanol preserved the zoospores slightly better than the Lysis Buffer, it had a significant inhibitory effect on the DNeasy PowerSoil Kit. Thus, each preservation medium presents their own benefits and limitations, and the application depends on the DNA extraction method being applied.

The DNeasy PowerSoil Kit-Lysis Buffer method delivered significantly better results over all the days and was the method of choice for this project. It would be recommended to process samples as quickly as possible, preferably within three days of collection; to prevent great losses in DNA. Samples should preferably not be left for longer than a week before processing, when using the Longmire's Lysis Buffer. For 75% of the samples, variances in Ct-values were directly linked to increased preservation times; thus, future quantification of field samples may become less accurate the longer the samples are preserved, regardless of the preservation medium. It is therefore recommended to process samples as soon as possible after collection in order to obtain the most accurate results. The Heat Lysis-Ethanol method presented the most consistent results overall over time, and no significant differences could be observed between any of the days. This method combination is also the most cost-effective of all the methods tested. Additional cleaning kits could possibly be applied to this method to improve the Ct-values in the future. Additional studies have also shown that improving the qPCR phase of a study can compensate for the shortcomings of the extraction method, such as increasing the number of qPCR replicates in the study (Piggott, 2016). This can be tested in future studies if a more cost-effective alternative would be preferred.

CHAPTER 4: MOLECULAR ASSAY DESIGNS AND OPTIMISATION

4.1 VALIDATION OF A TAQMAN ASSAY TO DETECT *B. DENDROBATIDIS*

When validating an assay, it is crucial to determine the lowest limit of detection (LOD). From previous studies the LOD between assays can vary significantly (Roussel *et al.*, 2015), and has to be determined for each assay. This may ultimately determine whether the assay is sensitive enough to be utilized for its intended purpose – in our case, the application of an eDNA assay. Knowing the LOD of an assay will assist in preventing false data. Real-time PCR assays are considered one of the most popular detection methods for eDNA, due to its cost-effectiveness and its ability to properly set standards (Langlois *et al.*, 2021). To develop a robust detection method for eDNA, multiple factors must be considered; including the sensitivity of the assay, the specificity of the primers to detect the target organisms, and primer efficiency. Contamination from nucleic acids can potentially cause false positives at lower concentrations. Thus, it is important to understand what the lowest limit of detection (LOD) for the developed assay is; as well as to include non-template controls (NTC's) to ensure that when amplifications occur, they are true amplifications and not false positives.

By analysing the performance characteristics of an assay, a criterion can be set which can be applied for results interpretation. Some studies are very stringent when setting their standard criteria, such as in the study conducted by Kirshtein *et al.* (2007) on *B. dendrobatidis*, which stated that any Ct-value above 35 was considered negative and excluded whether all PCR replicates amplified or not. Previous unpublished studies conducted on ventral swabs of amphibians at the Herpetological Health Lab (HHL), deemed Ct-values above 37 as negative. These were, however, samples from swabs, and a different standard may need to be set in the case of eDNA.

According to MIQE guidelines to determine the LOD, 95% of the samples should amplify per concentration in the standard curve, but this is also considered extremely strict for eDNA assays (Hunter *et al.*, 2017). Some studies follow the guidelines set by the MIQE because it delivers results with the highest level of confidence, but limits the protocol regarding the low DNA copy numbers (Guan *et al.*, 2019) – which is often the case in eDNA. The lowest limit of detection according to MIQE guidelines does not necessarily mean that Ct-values below this limit are false positives. It implies that values amplified below this threshold are less likely to be amplified in multiple replications. Thus, the repeatability of the sample to produce a positive result is reduced at lower concentrations, and may be excluded or deemed negative (Kralik & Ricchi, 2017). When the concentration of DNA is very low, as for eDNA, variation from pipetting may even result in no template being present in a sample during the qPCR step. Thus, samples that are expected to have low concentration may potentially not comply with the MIQE standards, and many less stringent methods have been developed for eDNA assays (Hunter *et al.*, 2017).

Studies utilising eDNA tend to follow their own method for determining the cut-off point as well as the LOD, and various approaches have been used in previous studies. In a study conducted by González *et al.* (2021), a sample was considered positive if both PCR replicates amplified and had a Ct-value below 40. In the study by Roux *et al.* (2020) the LOD was set as the concentration where 50% or more of the samples delivered a positive Ct-value. Other studies require as little as a single positive amplification out of three to eight replicate qPCR samples, and run twice on the machine to be considered positive (Rees *et al.*, 2014, Biggs *et al.*, 2015). In another two studies, the lowest detection limit was determined as where at least one well for each replicate sample delivered a positive value in the qPCR assay (Takahara *et al.*, 2013, Agersnap *et al.*, 2017). The LOD has also been defined as the

lowest level where one or more replicates of the qPCR assay delivered a positive amplification (Davison *et al.*, 2019).

There is a difference between the LOD of an assay and the Lowest Limit of Quantification (LOQ). The LOD refers to the lowest number of DNA copies that can be amplified with a level of confidence, whereas the LOQ refers to the level at which a sample can be confidently quantified with a high level of certainty and accuracy (Forootan *et al.*, 2017, Davison *et al.*, 2019). Both these factors are important in eDNA diagnostics. The LOQ tends to vary between studies, some referring to the CV% of the samples to determine the lowest dilution within an acceptable range of variation (Forootan *et al.*, 2017). Others define it as the value where 100% of the samples in a dilution amplify (Agersnap *et al.*, 2017, Davison *et al.*, 2019, Roux *et al.*, 2020).

4.1.1 Methods

The specificity of the selected primers has already been tested extensively in multiple studies (Boyle *et al.*, 2004, Hooper *et al.*, 2007, Blooi *et al.*, 2013) and was therefore not tested during this project. However, due to qPCR machines often varying in readings, a serial dilution series was tested using a synthetic DNA to determine the efficiency of the primers for the specific qPCR machine. The cut-off points for the Ct-values, LOD and LOQ of the assay was also determined using the dilution series.

A 742 bp gBlock fragment was designed to include the *B. dendrobatidis* gene region of interest. The real molecular mass of the gBlock fragment was provided by the manufacturer and was used to determine the number of DNA copies present for standard curve development using a modified version of the formula of Conte *et al.* (2018).

(gBlocks® Total Molecular Weight) x (1 mol/6.02 x1023 molecules) = weight per copy.

Reconstitution of the lyophilized gBlocks fragment provided 1.31 x 10^{10} copies/µL of the target. Dilution of the stock standard was done in TE buffer to create a sub-stock that was used to prepare the standard curve for the qPCR assay. Preparation of the standards consisted of diluting the concentrated standard from 10^6 copies/uL to 10^0 copies/uL. Three separate dilution series were prepared and qPCR for each series was repeated four times, adding to a total of 12 Ct. values for each concentration. This was used to create a standard curve for assay analyses. Previous studies have shown that the TaqMan probe-primer set used in this study has a high efficiency level and a high level of reproducibility between different qPCR plates (Hyatt *et al.*, 2007, Blooi *et al.*, 2013).

Following the literature, Davison *et al.* (2019) and Takahara *et al.*, (2013) the Limit of detection (LOD) was determined where at least one of the qPCR replicates for all the samples in each concentration amplified; provided that the Ct value conformed to the trend line, as well as followed the theoretical trend line created to be the LOD. The Limit of Quantification (LOQ) was determined at the lowest concentration where 100% of the samples amplified.

4.1.2 Results

Amplification curves that crossed the threshold before 45 cycles were included in data analysis. A total of 84 reactions were completed. The slope was calculated as -3.352 (Fig. 4.1). This calculated to an assay efficiency of 98.8% and a R^2 value of 0.9461.

Three of the twelve qPCR replicates for 1 DNA copies/µL amplified. These values were all above 37 Ct but did not conform to the theoretical standard curve (Table 4-1). Only one of the 12 qPCR replicates

for 10 copies/µl delivered a Ct-value, and the Ct-value was like those observed for the 1 copy per µL concentration. Thus, more samples amplified at a lower DNA copy number, and presented similar Ct-values, all above 37 cycles. For this reason, these values were considered unreliable.

At a concentration of 5 copies/µL, 25% of the samples amplified and was established as the LOD. The LOQ was determined as 5000 copies/ µL.

TABLE 4-1: Summary of the Ct values and amplification success of all the qPCR reaction the synthetic DNA dilution series

Template concentration (copies/µL)	Average Ct value (SDEV)	Amplification success (%)
5 x 10^6	21.41 (0.78)	100
5 x 10^5	24.18 (0.27)	100
5 x 10^4	27.71 (0.48)	100
5 x 10^3	32.13 (0.48)	100
5 x 10^2	35.93 (1.66)	75
5 x 10^1	37.36 (1.66)	8
5 x 10^0	38.73 (0.56)	25

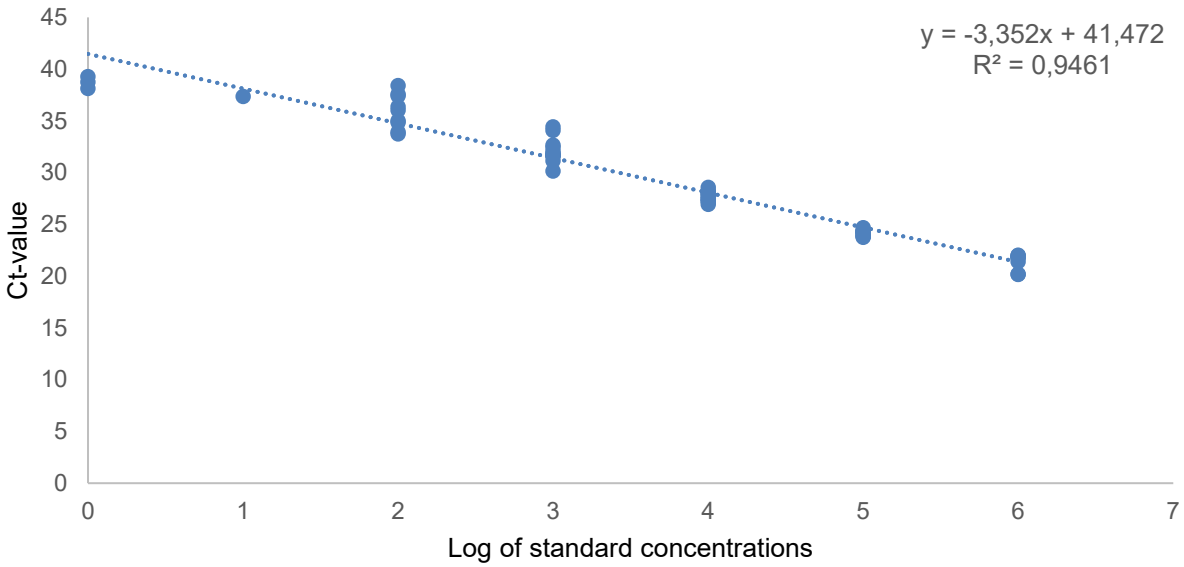

FIGURE 4-1: Graph showing the efficiency of the TaqMan probe-primer set used to amplify synthetic DNA containing the genome sequence of *Batrachochytrium.dendrobatidis*

4.1.3 Discussion

Samples at concentrations from and below 1000 copies/µL demonstrated a great increase in variability. Due to subsampling errors that often occur at lower DNA concentrations, the variability level between samples is expected to increase after 30 Ct-values.

Due to the expectation that eDNA concentrations are low, it is important to know the limit of detection to avoid false results. False amplification can be caused by background fluorescence being too high or non-specific amplifications, such as primer dimer formations (Hunter *et al.*, 2017). False positives can also be identified when samples with specific concentrations do not follow the expected trend line of a graph. This was observed in the raw data for the synthetic DNA dilution series at the lowest concentrations, where the Ct values were lower than the expected trend. In addition, more samples amplified at the lowest concentration compared to the second lowest concentration. None of the NTC's amplified on any of the runs, and samples were prepared from the lowest concentration to the highest, using separate strip tubes for each concentration, which provides confidence that the amplifications were unlikely to have been caused by the cross-contamination of samples.

4.2 TAQMAN ASSAY DESIGN TO DETECT *APHANOMYCES INVADANS*

With the increased awareness regarding the biosecurity of our nation's aquatic animal populations, and the consequent concern regarding a possible outbreak of foreign animal diseases, the rapid and accurate diagnosis of aquatic animal diseases is becoming increasingly important to both wild and captive populations of aquatic animals. Valid data and test results are indispensable; for the identification of disease, the verification of the health status of aquatic animal populations, and to inform epidemiological studies.

Quantitative polymerase chain reaction (qPCR) is quickly replacing other traditional methods for diagnosing potential pathogens in aquatic animal populations, due to the speed and robustness of the technology (Purcell *et al.*, 2011). Diagnostic assays must perform consistently and reliably over time to reduce the inevitable variability introduced by the application of these assays by different operators – often from different laboratories – who make use of varying analyte matrices that may differ in terms of sample origin and quality (Purcell *et al.*, 2011). Therefore, sufficient validation of any diagnostic assay is required prior to its application.

Assay specificity is the capacity of the assay to detect a genomic sequence that is unique to a targeted organism, and to exclude all other known organisms that are potentially cross-reactive. The nuclear ribosomal internal transcribed spacer region (ITS1-5.8-ITS2) is known as a universal DNA barcode marker for fungi and fungus-like organisms (Schoch *et al.*, 2012b). Previous studies have shown that analyses of the internal transcribed spacer (ITS) regions of rDNA provide a useful means of differentiating species of the related genera *Saprolegnia* and *Achlya* (Lilley *et al.*, 2003b, Greeff-Laubscher *et al.*, 2019).

Inclusivity is the capacity of an assay to detect several strains of a species. It characterises the scope of action for a screening assay. To date only one genotype for *A. invadans* has been recorded (Lilley *et al.*, 2003b, Diéguez-Uribeondo *et al.*, 2009, Iberahim *et al.*, 2018a, Huchzermeyer *et al.*, 2018), and therefore only one genotype strain was used during this study for the design of a TaqMan probe assay to detect *A. invadans.*

Analytical sensitivity represents the smallest amount of the target that can be measured in a biological sample. The limit of detection (LOD) is the measure of the analytical sensitivity of an assay. The LOD is the estimated amount of target in a specified matrix that would produce a positive result for at least a specified percentage of the time (OIE, 2017).

Epizootic Ulcerative Syndrome (EUS) is a seasonal epizootic disease of freshwater and brackish water fish caused by the Oomycete *A. invadans*. In South Africa, EUS was first isolated from the Palmiet and Eerste Rivers in the Western Cape in 2008. Subsequently the disease has been isolated from all

provinces except KwaZulu-Natal and the Eastern Cape. Surveillance efforts for this disease rely on accurate diagnostics. The demonstration of mycotic granulomas in histological sections of affected tissues and organs; isolation and culture of the causative organism from an infected fish; and the detection of *A. invadans* DNA in infected fish tissue, are all currently recognised as suitable diagnostic tests by the OIE. All these methods provide data regarding the presence or absence of the pathogen but have very little value for the quantification of the pathogen in the sample to estimate infection load or intensity of the infection in the infected host. The most useful method for quantification would be a method such as real-time PCR or qPCR. However, such methodology is not currently available for the detection of *A. invadans,* either from host tissues or from the environment (Oidtmann, 2012).

4.2.1 Methods

4.2.1.1 Assay design

Oligonucleotide TaqMan probe and primer (assay) design is a critical element in the experimental design process for any real-time qPCR experiment. The nuclear ribosomal internal transcribed spacer region (ITS1-5.8-ITS2) is known to be the universal DNA barcode marker for fungi, and was therefore chosen to be the target region (Schoch *et al.*, 2012a). Available data sequences of closely related species are most abundant for this region. The genetic sequence of the nuclear ribosomal internal transcribed spacer region between base pairs 1781 and 2428 (ITS1-5.8-ITS2) of *A. invadans* (Genbank accession number AF396684) of Vandersea *et al.* (2006), was aligned against sequences of closely related species that were downloaded from Genbank. MEGA X and MAFFT software (Berkley Software Distribution, University of California; version 7.221), and the L-INS-I option was used to align sequences. See Table 4-2 for species included in the alignment. Based on the *A. invadans* AF396684.1 sequence, the region between base pairs 1781 and 2428, was identified as the region of interest.

TABLE 4-2: List of closely related species included in the multi-species alignment.

Species name	Genbank Accession number	Isolation source	Reference
Aphanomyces invadans	AF396684.1	Menhaden fish	Vandersea *et al.*, 2006
A. frigidophilus	AY647192.1	Japanese char eggs	Unpublished
A. astaci	GU320237.1	*Astacus astacus*	Makkonen *et al.*, 2011
A. salsuginosus	AB510350.1	*Salangichtys microdon*	Takuma *et al.*, 2010
A. stellatus	AY455774.1	Water	Unpublished
A. laevis	AY283648.1	Mud	Unpublished
Phragmosporangium uniseriatum	KT935287.1	Soil	Unpublished
Achlya bisexualis	AY647189.1	Unknown	Unpublished
Saprolegnia parasitica	AY455777.1	Ayu fish	Unpublished
Saprolegnia ferax	JX087995.1	Water	Unpublished
Saprolegnia diclina	AY455775.1	Coho salmon	Unpublished
Achlya oblongata	LC149928.1	Asian seabass fry	Lau *et al.*, 2018
Leptolegnia chapmanii	KU896917.1	Unknown	Montalva *et al.*, 2016

Sci-Tools Primer Quest tool was used to design a TaqMan probe and primer sets based on the identified sequence region. A total of 10 TaqMan probe and primer sets were designed, of which 5 sets were identified for further screening (Table 4-3). An elimination process was used based on a more stringent criterion to narrow down to only 2 TaqMan probe and primer sets. These parameters are listed in

Table 4-4. The TaqMan probe and primer sets B and C were the best fit for the criteria and were thus chosen for further research application.

TABLE 4-3: Five TaqMan probe-primer sets first identified to undergo more stringent selection criteria.

	Type	Sequence	Sequence length (bp)	Amplicon length (bp)
A	Forward Primer	TCATTGTGAGTGAAACGGTG	20	85
	Probe	CGCCATTTAGAGGAAGGTGAAGTCGT	26	
	Reverse Primer	GTTCACCTACGGAAACCTTG	20	
B	Forward Primer	TCTTTATAAGGCTTGTGCTGAG	22	102
	Probe	TTTCTTGCGAAACCTTCGGCTAGC	24	
	Reverse Primer	GGCTAAGGTTTCAGTATGTAG	21	
C	Forward Primer	CGTCTGGAAGAGGTTTGTAGTAG	23	93
	Probe	TGCGGAGTGAGATAGTGTAATACTGGTGT	29	
	Reverse Primer	GTTTCCCAATTTGCTTCCGTATAG	24	
D	Forward Primer	TCGGCACAGGTAAACAACATA	21	105
	Probe	CGTCTGGAAGAGGTTTGTAGTAGAAGGC	28	
	Reverse Primer	AGACACAAGCACACCAGTATT	21	
E	Forward Primer	TCGGCACAGGTAAACAACATA	21	110
	Probe	CGTCTGGAAGAGGTTTGTAGTAGAAGGC	28	
	Reverse Primer	CGTATAGACACAAGCACACCA	21	

TABLE 4-4: Criteria used to identify 2 TaqMan probe-primer sets for further research application.

Overall	Probe	Primer
At least 1bp between primers and probe sequence	Melting temperature between 68°C and 70°C	Melting temperature between 59°C and 65°C
Amplicon size between 85 and 150bp	Less than 30 bases	Between 17 and 30 bases per primer sequence
	G-C composition 20%-80%	5' end stability and 3' end specificity, in other words 5 Nucleotides at 3' of each primer should not have more than 2G and/or C bases

Each TaqMan probe and primer set was added to the original alignment to ensure specificity among closely related species. Finally, to ensure species specificity during the desktop phase of the study, candidate primers (individually and in sets) were blasted against the GenBank, using the database provided by the online service of the National Centre for Biotechnology Information (http://www.ncbi.blast.nml.nih.gov/BLAST). Set B targets the ITS1 region and amplifies 102 bp, while TaqMan probe and primer set C targets the ITS2 region with an amplicon size of 93 bp.

4.2.1.2 Assay optimisation

Prior to specificity and sensitivity analysis, both primer sets were used to amplify nucleic acid from samples infected with *A. invadans*. Amplified products were sequenced to confirm amplification of the correct target. Following confirmation of the amplicons, different concentrations of probe and primer were tested. At first the probe concentrations were kept consistent at 100 nM per reaction while the primer concentrations varied from 100 nM to 500 nM per reaction, with increments of 100 nM. Following this step, the optimised primer concentration was kept consistent and paired with various probe

concentrations ranging from 100 nM to 250 nM with increments of 50nM. This was done at 2 different template concentrations. The optimum primer and probe concentrations were chosen based on the lowest average Ct. value, with the least variation between duplicates.

4.2.1.3 Assay specificity

Potential cross reactivity from closely related oomycetes were used to amplify oomycetes encountered in the freshwater environment. Freshwater Oomycetes were collected by baiting, enrichment, or direct isolation. The baiting method comprised 0.1 g autoclaved hemp seeds in plastic histology cassettes. The cassettes were submerged in the natural water bodies and retrieved after 48 hours. Upon collection, the cassettes were placed in honey jars and submerged in water from the sampling site. The honey jars were transported back to the laboratory where the hemp seeds containing growing hyphae were processed immediately for culture and DNA extraction. Although similar, the enrichment method comprised the collection of a water sample from a natural water body. The water sample was then transported back to the laboratory. The water samples were inoculated with autoclaved hemp seeds and left to incubate at room temperature, under sterile conditions. After 7 days of incubation, seeds containing growing hyphae were processed for culturing and DNA extractions. In addition to these, the oomycete *Achlya bisexualis* which was isolated directly from an infected fish, was also included in the reference panel (Greeff-Laubscher *et al.*, 2019)(Greeff-Laubscher *et al.*, 2019).

Hemp seeds containing growing hyphae were randomly divided into two groups. The first group was placed into a petri dish containing sterile water with fresh autoclaved hemp seeds and left to incubate at room temperature for culturing purposes. The second group was used to extract total genomic DNA.

The Petri dishes containing sterile water and hemp seeds were screened daily for actively swimming zoospores. When zoospores were observed, single spore isolations were conducted. A volume of 80-100 µL of zoospore suspension was transferred onto two 1.5% water agar plates and one Potato Dextrose agar (PDA) plate and spread using a glass plate spreader. Plates were incubated overnight at room temperature. Following incubation, 8 single spores or hyphae tips were transferred onto 2 Glucose Peptone Agar (GPA) plates (4 spores/ hyphal tips on each plate). After 2 days of incubation at room temperature, all 8 colonies were examined and transferred onto a new PDA plate. Following one week of incubation, total genomic DNA was extracted for molecular characterisation. Single spore cultures were maintained on PDA plates.

Total genomic DNA was isolated from all single spore cultures using a simple heat-lyses method (Greeff *et al.*, 2012). For initial cell disruption, samples were hand-homogenised for 1 min 30 sec using a handheld motorized pellet pestle (Sigma Aldrich, Cat. # Z359971). A universal fungal primer set (ITS5 and ITS4) was used to amplify the ITS1-5.8-153 ITS2 rDNA region for all isolates (White *et al.*, 1990). Reaction mixtures (total volume of 25 µL) consisted of 12.5 µL Taq DNA polymerase 2× Master mix (Amplicon PCR Enzymes & reagents, cat. No. #A140303) and 0.5 µL (0.25 µM) of each primer.

Amplification was conducted using the Labnet Thermal Cycler (Labnet International, Inc.) and consisted of an initial denaturation of 5 min at 94°C, followed by 35 cycles of 45 seconds at 94°C, 45 seconds at 51.1°C and 1 min at 72°C with a final extension of 7 min at 72°C. The amplified PCR products were analysed by agarose gel (1%) electrophoresis to verify fragment size. PCR products were sequenced using a BigDye Terminator Cycle Sequencing Kit (Applied Biosystems) and ABI3730xl Genetic Analyzer (Applied Biosystems), according to the sequencer manufacturer's instructions. Each sequence was edited and assembled using the software MEGAX (Kumar *et al.*, 2018). Homology searches were carried out using the BLASTN algorithm (Altschul *et al.*, 1990) provided by the Internet service of the National Centre for Biotechnology Information (https://blast.ncbi.nlm.nih.gov/Blast/)

4.2.1.4 Assay performance and sensitivity

A 781 bp gBlock fragment was designed to include the fragment of the ITS gene spacer region that is amplified by the primer set C (MRLaphn2F and MRLaphn2r). Copy numbers were calculated the same as previously done, and dilutions of the stock standard were prepared as before. Preparation of the standards consisted of diluting the concentrated standard from 10^6 copies/uL to 10^0 copies/uL.

Three independent dilution series were prepared, and each dilution series was run in triplicate. This was repeated six times; and in addition, another two runs were included using the same dilution series, but each with four replicates; adding to a total of 52 measurements for each concentration. The optimum primer and probe concentrations were used as it was determined in the previous step. Therefore, a total reaction volume of 25 µL containing 5 µL DNA template, 12.5 µL Taq DNA polymerase 2× Master mix (Amplicon PCR Enzymes & reagents, cat. No. #A140303), 300 nM of forward (Primer set C-MRLaphn2F) and reverse (Primer set C-MRLaphn2r) primers, and 200 nM TaqMan® probe. The PCR program consisted of 5 min at 95°C to activate the polymerase and denature the template DNA, followed by 45 cycles of 15 s at 95°C and 60 s at 60°C. Following the run, Bio-Rad CFX Maestro 1.0 software was used to analyse the data. Prior to analyses, fluorescence drift correction was applied. The baseline of each run was manually adjusted as follows: amplification curves were viewed in a log format to determine which reaction in the assay emerged earliest from the baseline. This reaction was used to calculate the stop value for the baseline by subtracting 2 from the value where amplification started (e.g. baseline stop value = X-2). This stop value was applied to all the reactions across the plate. The start value of the baseline was left at 2, the default value as calculated by the software. The threshold was auto calculated by the software, based on the adjusted baseline values. The average Ct. values vs. the log of concentrations was used to determine the assay efficiency. The efficiency was calculated by using the following formula:

$$E=-1+10^{(-1/slope)} \times 100.$$

LOD and LOQ were calculated as previously explained.

4.2.2 Results

Two sets of TaqMan probe-primer sets (MRLaphn1 for probe set B, MRLaphn2 for probe set C) were used to amplify *A. invadans* from historically known positive samples. Amplified products were sequenced to confirm amplification of the target region.

4.2.2.1 Assay optimisation

TABLE 4-5: Summary of average Ct values when comparing different primer concentrations and different probe concentrations.
*Concentrations chosen to be used for further validation

4.2.2.2 Assay specificity

A panel of single spore cultures was used to evaluate the assay specificity for TaqMan probe-primer sets. This panel of cultures included fungi that were previously isolated from the environment, as well as oomycetes closely related to *A. invadans* (Table 4-6).

TABLE 4-6: List of fungi and oomycetes used to screen two selected TaqMan probe-primer sets and showing where amplification took place.

Probe concentration (nM)	Primer concentration (nM)	Template: 10^4 Average Ct. (STDEV)	Template: 10^3 Average Ct. (STDEV)
100	100	39.5 (±2.800)	37.31 (±6.675)
	200*	26.75 (±0.007)	31.19 (±0.197)
	300	26.48 (±0.155)	30.97 (±0.254)
	400	26.31 (±0.155)	31.4 (±0.381)
	500	26.43 (±0.120)	31.11 (±0.183)
100*	200	29.90 (±0.120)	31.33 (±0.657)
150		26.65 (±0.169)	31.61 (±0.586)
200		26.48 (±0.183)	31.37 (±0.077)
250		26.405 (±0.106)	31.42 (±0.678)

Species name	Order	MRLaphn1	MRLaphn2
Saprolegnia ferax	Saprolegniales	X	X
S. australis	Saprolegniales	X	X
S. aenigmatica	Saprolegniales	X	X
S. parasitica	Saprolegniales	X	X
S. longicaulis	Saprolegniales	X	X
Achlya bisexualis	Saprolegniales	✓	X
A. crenulata	Saprolegniales	X	X
A. prolifera	Saprolegniales	X	X
Achlya species	Saprolegniales	X	X
Unkonwn Oomycete	Saprolegniales	X	X
Halioticida noduliformans	Haliphthoraceae	X	X
Batrachochytrium dendrobatidis	Rhizophydiales	X	X
Aphanomyces astaci	Saprolegniales	✓	X
Fusarium keratoplasticum	Hypocreales	X	X
F. falciforme	Hypocreales	X	X
F. fujikori	Hypocreales	X	X
Positive control samples:			
EUS infected tissue sample 1	Saprolegniales	✓	✓
EUS infected tissue sample 2	Saprolegniales	✓	✓
EUS infected tissue sample 3	Saprolegniales	✓	✓

Taqman probe set B showed some cross-reactivity with other oomycetes, including *Achlya bisexualis* and *Aphanomyces astaci*. The lack of exclusivity is likely to yield undesirable false positive results. Consequently, analytical sensitivity and assay diagnostic performance characteristics would only be assessed for Taqman probe set C, which only amplified *A. invadans* DNA.

4.2.2.3 Assay performance and sensitivity

Amplification curves that crossed the threshold before 45 cycles, were included in data analysis. A total of 364 reactions were completed. The slope was calculated as -3.3425 (Fig. 4.2). This calculated to an assay efficiency of 99.148%, and a R^2 value of 0.894. The R^2 value is the square of the correlation between the response values and the predicted response values; in other words, it measures how

successful a fit is by explaining the variation of the data. An R^2 value of >0.980 indicates good confidence in correlating Ct. value and target copy number (Bustin & Huggett, 2017).

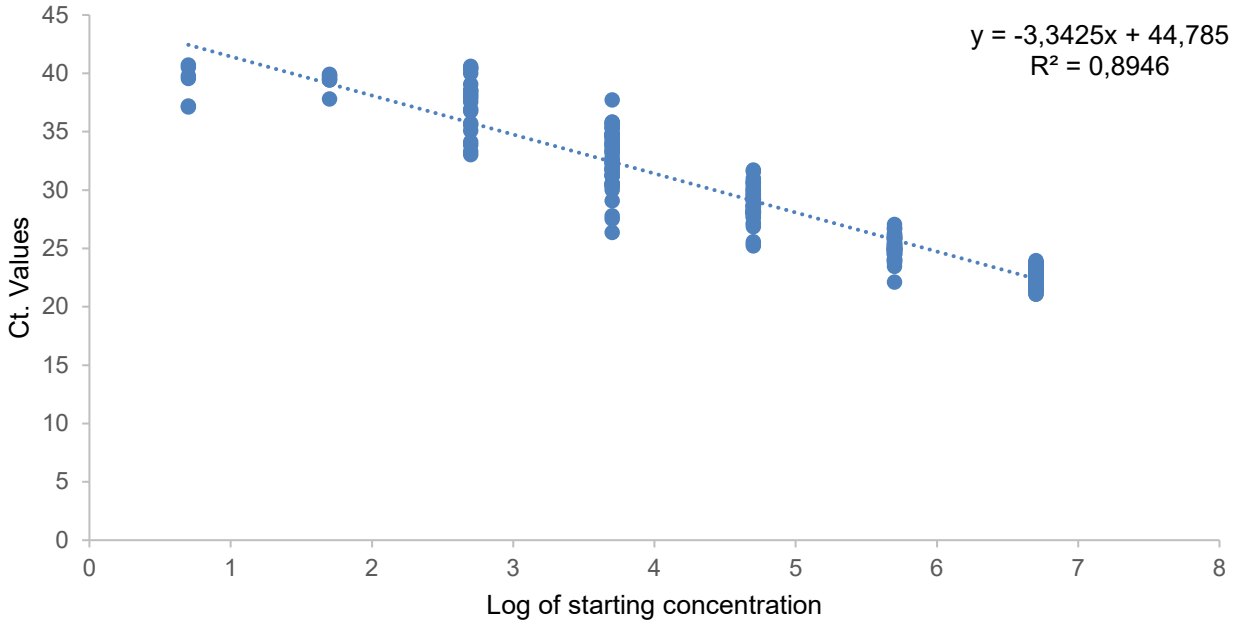

FIGURE 4-2: Graph showing the efficiency of the novel primer-probe set C (MRLanph2)

TABLE 4-7: Summary of the Ct values and amplification success of all the qPCR reaction the synthetic DNA dilution series

Template concentration (copies/µL)	Average Ct value (SDEV)	Amplification success (%)
5 x 10^6	22.70 (0.78)	100
5 x 10^5	25.14 (0.92)	100
5 x 10^4	28.81 (1.14)	100
5 x 10^3	32.75 (2.33)	100
5 x 10^2	37.10 (2.43)	33.46
5 x 10^1	39.40 (0.72)	13.46
5 x 10^0	39.22 (1.47)	13.46

The LOD was determined as 5 copies/µL, while the LOQ was determined as 5000 copies/µL.

4.2.3 Discussion

Assay design, optimisation and validation can be seen as the cornerstone of any research project designed to detect nucleic acids. Ideally the assay should be reliable and repeatable. Reliable PCR assays require good primers and probes, which means absolute specificity. It is crucial to properly validate newly designed assays (Bustin & Huggett, 2017). Initially two primer-probe sets were designed and synthesised. Following initial PCR reactions, it was confirmed that both primer sets amplified the correct targeted regions, and this was supported by amplicon sequences and melt curve analysis.

Analytical specificity was determined when both sets were screened against a panel of fungal DNA extracted from single spore cultures. Only one primer-probe set (Set C) was specific enough to justify further optimisation and validation. Unfortunately, primer-probe set B cross-amplified two closely related oomycete species.

Assay optimisation focused on the primer and probe concentrations per reaction. The optimum concentrations were determined as 200 nM of TaqMan probe per reaction, and 300 nM primer concentration in each reaction.

Robust and precise qPCR assays correlate with high assay efficiency. A standard curve generated from qPCR data with a serial dilution of the target template is used to determine the assay efficiency. A well optimised assay should have an efficiency close to 100%, with a high reproducibility between replicates (Johnson *et al.*, 2013). The efficiency of this assay calculated to 99.14%, which falls well within the suggested parameters of an efficient assay. An efficiency of 90-110% is considered acceptable. The R^2 value of the graph was also determined along with the efficiency to determine the reliability of the graph.

4.3 INTERNAL CONTROL DETECTION

4.3.1 *Amietia* spp.

Due to the lack of available real-time PCR assays in the literature for the two chosen hosts, new assays had to be designed. As this did not form part of the outcomes of this study; instead of spending time and resources towards designing novel TaqMan probe-primer sets, custom designed assays were ordered from a commercial company.

According to the manufacturer (YouSeq Ltd), the primers would successfully amplify the following species from the Pyxicephalidae family, with distributions obtained from Channing and Rödel (2019): *A. delalandii* (western Mozambique, South Africa, Lesotho, Zimbabwe, Malawi, and Zambia); *A. fuscigula* (South Africa);*A. vandijki* (South Africa); *A. vertebralis* (Lesotho and South Africa); *A. poyntoni* (Lesotho, South Africa, and Namibia); *A. johnstoni* (Malawi); *A. moyerorum* (Malawi and Tanzania); *A. ruwenzorica* (Uganda and Democratic Republic of Congo); and *A. wittei* (Kenya, Tanzania, and Uganda).

This assay was validated for the intended use. A serial dilution was prepared and amplified following manufacturing instructions.

The Genus *Amietia* was chosen as the internal assay control, because 1) *Amietia* is widespread in South Africa and abundant where they occur (even occurring throughout sub-Saharan Africa), thus maximizing the likelihood of encountering a target amphibian host; 2) the species within the genus *Amietia* seldom occur sympatric and their distributions have restricted overlap, thus by being able to amplify multiple species, we ensure a country-wide application of the assay; 3) *Amietia* tadpoles are slow developers, with some species taking more than a year to reach metamorphosis, thus enabling us to sample eDNA throughout the year as opposed to most other species that display restricted temporal larval development; 4) at least three of the *Amietia* species, *A. delalandii, A. fuscigula* and *A. vertebralis,* are known to be highly susceptible to *B. dendrobatidis* infection, and are therefore good host species to work with when building epidemiological models (Weldon & Du Preez, 2006, Smith *et al.*, 2007, Conradie *et al.*, 2011, Griffiths *et al.*, 2018).

4.3.1.1 Assay performance

This assay was designed and assembled in a qPCR kit for detection and quantification of aquatic river frogs, *Amieta* spp. DNA from a variety of sources. Total genomic DNA was extracted from four *Amietia* species and one outgroup host species, namely *Strongylopus grayii* (Clicking stream frog), to evaluate the analytical specificity of the assay.

Tissue samples were obtained from old samples collected and stored by the African Amphibian Conservation Research Group at the North-West University. Samples were stored in a freezer at -80°C, and small aliquots were used for DNA extractions. The tissue samples were from different body parts of the specimens and differed in size and weight. They were finely cut using scissors that were sterilised with 70% ethanol and flamed between each sample. Extra care was taken to avoid cross-contamination. DNA was extracted according to the Heat Lysis method (Greeff *et al.*, 2012). The Heat Lysis method was applied for the DNA extraction because the tissue samples were stored in 95% ethanol, which has shown to have an inhibiting effect on the DNeasy PowerSoil Kit, as previously discussed.

The assay showed satisfying results by amplifying all *Amietia* spp. tested, while the assay failed to amplify the outgroup tested (Table 4-8). To confirm that cross-amplification wouldn't occur with closely related species, *Strongylopus grayii* was chosen as an outgroup species because it belongs to the same family as *Amietia*, Pyxicephalidae. Additionally, *S. grayii* shares the same habitat as *Amietia* throughout most of the country and is therefore ecologically relevant to the implementation of the assay.

TABLE 4-8: List of host species used to screen the *Amietia* spp. qPCR assay.

Species name	Family	Amplification success
Amietia fuscigula	Pyxicephalidae	✓
Amietia delalandii	Pyxicephalidae	✓
Amietia poyntoni	Pyxicephalidae	✓
Amietia vertebralis	Pyxicephalidae	✓
Strongulopus garyii (outgroup)	Pyxicephalidae	X

The PCR assay to detect *Amietia* spp. (host of *B. dendrobatidis*) was validated by following the manufacturer's instructions. A positive control sample containing synthetic DNA with the genetic gene region of interest was used to create serial dilutions starting at 10^6 copies/µL diluted down to 10^1 copies/µL. Each series was run in duplicate. The slope was calculated as -3.3159 (Fig. 4.3). This calculates to an assay efficiency of 100.25%, with a R^2 value of 0.994 and the lowest detection limit of 100 copies/µL.

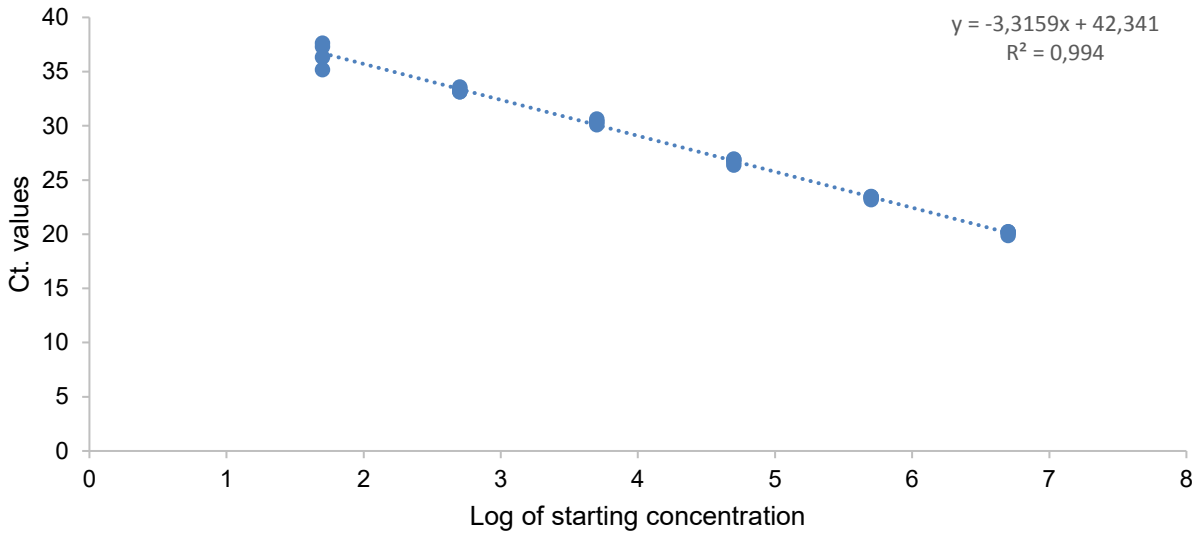

FIGURE 4-3: Logarithmic graph showing the efficiency of the *Amietia* spp. PCR assay.

4.4 LAMP ASSAY DEVELOPMENT AND OPTIMISATION

4.4.1 Methods

4.4.1.1 LAMP assay development

A sequence of all *B. dendrobatidis* lineages were used to design LAMP assay primers. These include IA042 and JEL197 (*Bd*GPL), TF5a1 and TRBOMB (*Bd*CAPE), KRBOOR331 (*BdAsia-1*), and KB45 (*BdAsia-2*). Sequence data was obtained from both the online National Centre of Biotechnology (http://www.ncbi.blast.nml.nih.gov/) and international collaborators. Primers were designed using Primer explorer v5. Over 500 possible primer sets were designed targeting the ITS gene region. A stringent criterion was applied, and the most theoretical set of primers were chosen according to the most optimal melting temperature, GC content and dG values. One primer set consists of six primers, varying from 18 to 42 bp per primer. Finally, to ensure species specificity during the desktop phase of the study, candidate primers (individually and in sets) were blasted against the GenBank, using the database provided by the online blast service of the National Centre for Biotechnology Information (http://www.ncbi.blast.nml.nih.gov/BLAST).

LAMP reactions take place at an isothermal temperature. The biochemistry of a LAMP reaction can easily result in false positives when an assay is run for too long. This is due to primers binding to themselves in the absence of target DNA. It is for this reason that the temperature at which the assay is performed is optimised, to ensure both optimal specificity and low to no risk for false amplification. A temperature range of 58-65°C was tested. Negative control samples were used for temperature optimisation. Each reaction tube consisted of 15 µL Optigene's Isothermal mastermix with a specialised fluorescent dye, 2.5 µL primer mix, 5µL total genomic DNA and 2.5 µL nuclease-free water.

4.4.2 Results

When running negative controls, it is determined which temperature will decrease the possibility of false positive detection, and which temperature is more favourable at an increasing speed. Both 63°C and

60°C degrees displayed false positive amplification after 60-90 minutes. In contrast, samples that were run at 58°C did not display any false positive amplification in the cycle duration. Therefore, the LAMP reaction would be further optimized and utilized for field and environmental DNA samples using 58°C.

CHAPTER 5: ASSAY VALIDATION

5.1 INTRODUCTION

Validation of the laboratory-tested assay requires collecting samples from the environment, using the chosen filtering system, and processing the samples according to the new assay. When collecting field samples, it is important to consider the time that the samples will be taken, as well as where they will be taken. Both aspects can influence the sensitivity of the overall assay. The concentration and distribution of eDNA is influenced by the physiology and spatial use of organisms, as well as the water movement (Goldberg *et al.*, 2016). Depending on water movement, a water sample collected at one point along the shore of a wetland may not be representative of the whole wetland. In the scenario where the aim is to apply the eDNA assay as a targeted approach such as in this study, it becomes important to understand the behaviour of the target organism, to ensure that samples are collected where the target organisms are most likely to be found. In some cases where the target organism is a pathogen, the life cycle of the pathogen becomes important, as well as the behaviour of the host. In the case of *B. dendrobatidis,* the host includes various frog species in South Africa, thus it would be more likely to find these at shallower waters, rocky pools, ponds, etc.

The species' distributional range, in this case for both the pathogen and the host, is important for the consideration of site selection. Distributional modelling can assist in better understanding species distribution. Geographic information systems (GIS) software creates the means of producing high quality as well as sophisticated models, aimed at establishing the possible distributional ranges of any given species. The success of GIS modelling is partly due to the understanding of the link between species distribution (abundance) and the environmental parameters/conditions. There is a strong correlation between environmental conditions and species distribution. A predictive model was created using species locality data that consists of geo-referenced coordinate data (collected in field work/studies), and environmental parameters. The resulting model was projected onto a habitat suitability model/map. Creating this map was a secondary objective in this study, to form the foundation of site selection. See the Honours dissertation for this part of the project in Appendix B.

The lineages *Bd*CAPE and *Bd*GPL have been associated with the most amphibian mortalities of all the known lineages, and both of these occur in South Africa (Ghosh *et al.*, 2021). Models of positive *B. dendrobatidis* samples determined that the pathogen mainly occurs in the coastal, as well as high rainfall regions in the eastern sections of the country (Tarrant *et al.*, 2013). High elevation, low temperatures, and high precipitation appeared to be the major drivers to the pathogen's prevalence. Many studies have previously been conducted on *B. dendrobatidis* within the Drakensberg area of the country (Griffiths *et al.*, 2018, Ghosh *et al.*, 2021). The Drakensberg Mountain in South Africa represents the highest escarpment in the country; and the climate is typically associated with colder temperatures and above average precipitation, compared to the rest of South Africa. The majority of frog species that have tested positive for *B. dendrobatdis* in the Drakensberg belong to the genus *Amietia,* and the three most common species were *Amietia hymenopys, Amietia delalandii* and *Amietia vertebralis*.

5.2 METHDOS

5.2.1 Site selection

The target localities for the collection of environmental DNA samples include Ncandu Nature Reserve, Soutpansberg, Blyde River Nature Reserve, and the Mont-aux-Sources region of the Drakensberg.

These sites are well monitored and well known for *B. dendrobatidis* presence, thus ideal for field validations.

FIGURE 5-1: Map showing the locations where samples were collected

To date, the sites for the Ncandu Nature Reserve and Soutpansberg conservancy localities have been sampled, and the samples were processed according to the methods below. A planned fieldtrip to the Mont-aux-Sources region of the Drakensberg at the end of 2022 did not transpire, due to adverse weather conditions and heavy rainfall, which is not well-suited for detecting *B. dendrobatidis* in water bodies.

TABLE 5-1: Summary of the field sampling at each location

Locality	Number of samples sites at each location	Date of sampling
Ncandu Nature reserve	3	April 2022
Soutpansberg	1	September 2022
Blyde River	2	11-14 January 2023

5.2.2 Sampling collection, processing, and amplification

5.2.2.1 Sample collection for eDNA detection

Water: Water samples were collected by utilizing a 250 mL analytical funnel filter cup (Thermofisher, USA). Collection of five water samples per site was conducted. Approximately 500 mL of water was filtered, or until the filter clogged. Consequently, filters were cut in half with sterile scissors and placed in 2-3 mL of lysis buffer solution contained within a falcon tube. Upon transport to the laboratory, the filter samples were stored in a -80°C degree freezer until further processing.

Rock swabs: Sterile dry swabs were used for the collection of eDNA from rock surfaces where frogs were visually seen around a water body/stream. Since *B. dendrobatidis* is an aquatic fungal species, damp rock surfaces were swabbed with 5 samples collected from each site. The swabs were stored in Eppendorf tubes for transport and stored in a -80°C freezer at the laboratory until further processing.

Sediment: Five sediment samples were collected from each site. This was conducted by sampling 0.5 m apart across the circumference of the water body for each sample. Approximately 0.3 g of sediment were collected for each sample and stored in Eppendorf tubes. Upon transport to the laboratory, the samples were stored in a -80°C freezer until DNA extraction.

5.2.2.2 DNA extraction

DNA extraction involved the use of the Qiagen DNeasy Powersoil Pro kit for environmental samples. Water filters required the additional step of aseptically cutting the filter into small pieces prior to the initialization of the manufacturers protocol. The cotton swabs and sediment samples did not require additional processing steps.

5.2.2.3 Molecular detection

For qPCR, an adapted method was used of the Taqman probe qPCR assay developed by Boyle *et al.* (2004) for *B. dendrobatidis* detection. A total of 25 µL was utilized for each reaction in an optical 96 well plate. Each reaction consisted of 0.5 µL (200 µM) forward and reverse primer, 0.25 µL (100 µM) MGB probe, 12.5 µL universal probes supermix, 6.25 µL water and 5 µL sample product. A Quantstudio 3 qPCR thermocycler was used with reaction conditions of 2 mins at 50°C, 10 min at 95°C, followed by 15 s at 95°C, and 1 min at 60°C for a total of 50 cycles. Afterwards, results were analysed in Quantstudio design and analysis software. The LOD for the primer was 100 copies/µL and the cut-off Ct-value was determined to be 37 cycles. Samples can be measured according to the values from the standard curve generated, but due to the LOQ of the primers being 1000 copies/µl, it is not recommended to quantify samples below this value.

5.2.3 Internal assay control

Amietia delalandii is an excellent target specimen due to its distribution throughout South Africa and low mortality rate, even when infected with *B. dendrobatidis.* This frog species has shown to survive high infection loads, and are often used in South Africa to determine the presence of *B. dendrobatdis* in the environment (Antwis & Weldon, 2017).

5.2.4 Cytology

Cytology was used as an external test to verify the eDNA results. Skin sloughs from the feet, hind legs and ventral pouch can be used to diagnose chytridiomycosis without the need for staining or tissue processing (Longcore, 2001). Similarly, the keratinised mouthparts of tadpoles can be used to prepare wet mounts for the purpose of diagnostic screening for *B. dendrobatidis* (Smith & Weldon, 2016). We used tadpoles of *Amietia* from sample sites to screen for *B. dendrobatidis* infection as an internal control for the infection status of a site. A dip net was used to sample tadpoles by sweeping among aquatic vegetation near the edges of water bodies. Tadpoles were euthanized prior to any tissue sampling procedures. Benzocaine was administered by immersing a tadpole, allowing the absorption of chemicals through the skin. Benzocaine (250 mg/L solution) was prepared by adding 25 mg benzocaine to 50 mL hot water, allowing it to cool, and adding a further 50 mL cold water. Sterilized dissecting equipment was used to excise the mouthparts (including labial papilla, kerotodonts and rostrodonts) for cytological screening for *B. dendrobatidis*. Tadpole mouth discs were placed on microscope slides in a drop of sterile water, with a coverslip examined for the presence of fungal thalli consistent with *B. dendrobatidis* using a compound microscope (100 x and 400 x magnification) (Weldon & Du Preez, 2006).

5.3 RESULTS

All the internal control samples amplified successfully. All the relevant assay controls passed.

TABLE 5-2: Summary of qPCR results for eDNA from different sites

	Ncandu site 1	**Ncandu site 2**	**Ncandu site 3**	**Soutpansberg**
Water	Not detected	Not detected	Not detected	Not detected
Sediment	Detected*	Detected*	Not detected	Detected*
Rock swabs	Not detected	Not detected	Not detected	Not detected
External *B. dendrobatidis* verification (Cytology)	Not detected	Detected	Not detected	Detected

*A site was called positive when at least one out of the samples amplified in less than 35 cycles

CHAPTER 6: CONCLUSIONS & APPLICATIONS

Detection of pathogens, parasites, plants, or any organisms from environmental samples via eDNA is an increasingly important tool with which to assess information about their occurrence through time and space. These detection assays have great application potential for determining infections of host organisms in connected habitats, for both pathogen monitoring and surveillance, and early warning systems for diseases. Before eDNA detections can be reliably interpreted and adopted for its purpose (e.g. surveillance and assessment of disease risk), there are many factors that require consideration such as appropriate method development and validation (Bass *et al.*, 2023). The World Organisation for Animal Health (WOAH) has established a list of notifiable diseases with the aim to lower the risk of these diseases spreading. Two of the fungal pathogens listed by the WOAH is present in South Africa and have had significant impacts on global amphibian and fish health. The first objective for our project was to develop and validate an eDNA assay for specific use in South African freshwater systems. For the development of this assay the pathogenic *B. dendrobatids* was used as the target organism. The second objective was to develop, optimise and validate a molecular assay to detect *A. invadans*. Both target pathogens have a wide distribution in South Africa and are associated with mass mortalities globally.

Overall, this project delivered substantial results that render the eDNA assay appropriate for wide-scale implementation. A new sampling assay to detect *B. dendrobatidis* from the environment in South African waters was developed and validated. Although this detection assay focused on a particular organism, the assay can be tailored to allow for the detection of any species with only a few minor modifications. A molecular assay to detect *A. invadans* was also designed and optimised.

The eDNA assay development was completed in four steps: sample collection, sample preservation, sample processing and target detection. A syphon pump method was the best way to filter water due to its efficacy while being practical and simplistic. Greater increases in Ct-values over time resulted from preserving samples in a Lysis Buffer as compared to ethanol-preserved samples. Moreover, 70% ethanol did not deliver significantly better results than the Lysis Buffer for any of length of preservation tested when the Heat Lysis method was used to extract DNA from samples. Thus, overall, both methods performed very similarly in terms of Ct-values over time and the preservation medium did not drastically affect the results for this method. When the DNeasy PowerSoil Kit was used in conjunction with the Lysis Buffer, significantly better results were obtained for all preservation times. We therefore recommend these steps as the method of choice for this project. The Heat Lysis method appears to be more reliable, delivers more consistent results within each matrix, is less time consuming, more cost-effective, and does not apply hazardous chemicals. For these reasons it is recommended that if commercial kits are not available and a crude extraction has to be used, that the Heat Lysis method is used.

Based on the results from both the synthetic DNA as well as the filter dilution series, a cut-off point of 37 Ct was selected for the most reliable results in field application for the detection of *B. dendrobatidis.* Although samples with as little as 5 DNA copies/µL amplified in our assay, a LOD for the synthetic DNA was determined at 100 DNA copies/µL, because more than 50% of the replicates for all the samples amplified. The LOD for the filter material test is approximately 10 zoospores per filter. This is more sensitive than the LOD reported by a previous study as 100 zoospores per filter when eDNA was extracted using the PowerSoil Kit (Brannelly *et al.*, 2020). Based on these *in vitro* results our assay is very likely to be sensitive enough for field application of *B. dendrobatidis* eDNA. The LOQ for the assay is 5000 copies/µL, which is a high value when the aim is to apply the assay to large open water systems. However, this assay would be best suited for application in smaller lentic water bodies or slow flowing

streams, such as those typically inhabited by amphibians. While the application of this assay to sediment samples has not yet been fully validated, preliminary results are very promising. It is therefore recommended that the biology and behaviour of the target and host species be considered when deciding on which sample types to utilise.

Basic conservation information, such as species occurrence, distribution, abundance, habitat requirements, and threats are not readily available for all species. Using environmental DNA increases the ability to detect and quantify biodiversity where conventional sampling and identification methods fail. Two broad approaches that have received the most attention in eDNA-based studies is targeted and non-targeted approaches; the latter is also known as metabarcoding. The main difference between these approaches is that a targeted approach uses species-specific primers to detect the DNA fragments of a single species within an environmental sample, while non-targeted uses universal primers to simultaneously detect countless DNA fragments from the widest possible range of species from multiple trophic levels. In this study the focus was to develop an assay to sample and extract eDNA from the environment. In addition, the final molecular assay that was applied to the extracted nucleic acid was a targeted assay, but a non-targeted assay could just as easily have been applied. This project used a targeted approach to refine and evaluate the assay, and this assay can be applied to research questions of conservation concern related to the detection of rare, cryptic, or endangered species, estimating species distribution, biomonitoring the health of ecosystems and determination of its dynamics, diet and tropic interactions and monitoring the biodiversity. Importantly, this assay can also be applied to routine screening within the aquaculture industry with the aim of early disease detection and prevention of epidemics, and regulatory authorities can use this assay in combination with a non-targeted molecular detection to determine and monitor microbial communities in water sources.

The new TaqMan probe primer set that was developed in this study to detect *A. invadans* showed similar potential, as it is the first ever qPCR assay developed to detect the causative agent of EUS, both nationally and internationally. Future studies should validate this assay in different matrices. The target amplicon is short; therefore, this assay should perform well in the application of eDNA assays, making it an ideal tool for early detection of EUS outbreaks in freshwater fish farms.

In conclusion, this project has created a solid foundation for eDNA application in South African waters and paves the way for a highly technological development in disease surveillance that adheres to the 3R’s of animal ethics by presenting a non-invasive method for sampling aquatic biodiversity.

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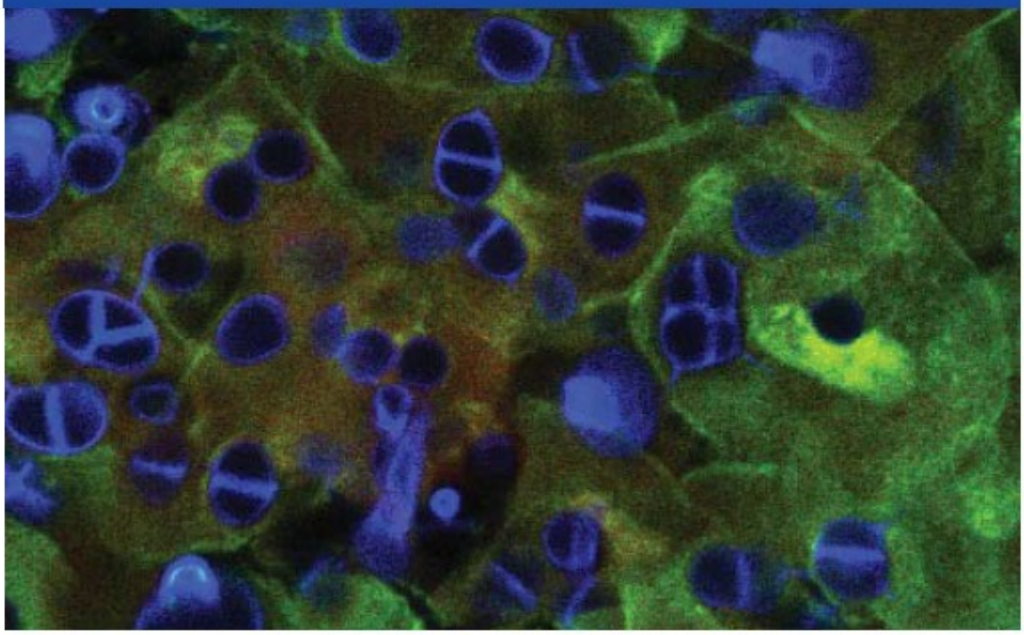
APPENDIX A:

POPULAR ARTICLE IN WATER WHEEL (JULY/AUG. 2020)

DISEASE RESEARCH

Diagnostics and aquatic emerging fungal diseases

History has shown that pandemics can have major impacts on populations and the environment. The current Covid-19 pandemic is turning the world upside down, and will undoubtedly leave its footprints on all sectors of life to some degree. So writes MR Greeff-Laubscher and C Weldon.



Laser scanning confocal micrograph of amphibian skin (green epidermal tissue) infected with Batrachochytrium dendrobatidis (blue fungal bodies).

The infection pathway and high infection rate of this causative virus affect our social lives, and even our behaviours are impacted and changed. A question we should be asking is if any of these adversities could have been avoided had we been more prepared? Or, even more fundamentally, is it at all possible to be prepared for dealing with the diagnosis of a pandemic?

What are the driving forces behind research in the field of emerging infectious diseases (EIDs) – is it engagement with current known diseases and their related strains, or is it lessons learnt from historic epizootics? In reality, it is incredibly difficult to prepare for EIDs, be it human or animal diseases, especially when the epidemiology and infection rates are not yet understood. This begs the question, how do we develop accurate and reliable diagnostic tools for EIDs if the infectious agent is not yet known? The current Covid-19 pandemic, caused by the infectious virus, SARS-CoV-2, is an example of how this zoonotic coronavirus became pandemic within just a few months. Infectious agents can include a variety of microbial organisms, including bacteria, viruses, fungal and fungal-like organisms.

Over the past two decades, there has been an increase in fungal diseases both in plant and animal (aquatic and semi-aquatic) populations. A record number of fungal and fungal-like diseases have now known to cause mass mortalities and species extinctions. Some examples include ***Batrachochytrium dendrobatidis***, the causative agent of amphibian chytridiomycosis; ***Aspergillus sydowii***, the causative agent of aspergillosis in marine coral; and ***Aphanomyces invadans*** and ***A. astaci*** causing epizootic ulcerative syndrome in fishes, and crayfish plague respectively.

In the face of unprecedented, rapid declines in global biodiversity, scientists have been investigating how ecosystems with altered biodiversity affect the provision of beneficial ecosystem services. Studies on Lime diseases, for instance, indicated that a greater diversity of hosts lower transmission risks to humans. One can deduct from this finding that a loss of biodiversity would likely result in an increase of infectious disease transmission to humans.

The impact of pandemics can be lowered with quick, reliable and accurate diagnostics. Once infected individuals have been diagnosed, management plans can be put in place to either control or eradicate the infectious agents. While population density and disease susceptibility are some of the major risk factors involved during disease outbreaks, another silent factor is asymptomatic individuals. During the absence of reliable diagnostics, the latter is often underestimated or remains undiagnosed within a population. This is especially true when diagnostic assays are dependent on clinical symptoms (in humans) or visible clinical signs (e.g. in animals).

Although it is referred to as a silent factor, we prefer to refer to it as ***ticking bombs*** because these individuals have the ability to infect other individuals in their environment, apparently unnoticed. If this would happen with a highly infectious disease, an epidemic can occur, resulting in high mortality rates in severely affected populations. To lower the effect of these ***ticking bombs*** on their populations, reliable diagnostic tools are required.

Most diagnostic protocols can be divided into three phases. These are: sampling, sample processing, and sample analysis. The saying "only as strong as the weakest link" is a good way to approach a diagnostic assay. If one of the steps is not reliable, the entire diagnostic assay becomes unreliable.

Validating each step of a diagnostic assay is therefore a prerequisite for achieving reliability. It bridges laboratory work and field application by assessing the application of a specific technique related to the intended use of that technique. The validation step truly reveals if an assay is good enough to perform to the anticipated outcome.

Validation of an assay can be time-consuming and is often neglected during the development of diagnostic (identification from a host) or detection (identification from environment) assays, especially when each step has to be validated. Using an unvalidated diagnostic assay lowers the confidence in the results. Such assays could either lead to the misinterpretation of negative results (false negatives) or an incorrect number of positive individuals (false positives) or sites reported, and therefore lead to inappropriate management of a pandemic.

The causative agent of the next animal pandemic and its impact on aquatic biodiversity is currently unknown to us; however in the midst of a changing climate it has been argued that emerging fungal infections will cause an enhanced abrasion of biodiversity, which spill over to human and ecosystem health. Just like certain bacteria and virus species, fungal and fungal-like organisms can be lethal to naïve species. ***Batrachochytrium dendrobatidis*** is a true panzootic, with a wide host range and the ability to survive outside the host. The global occurrence of this pathogen was facilitated by anthropogenic spread, which resulted in an estimated 90 amphibian species becoming extinct.

The first report of this pathogen on the African continent dates back to the early 1930s. Researchers are now busy using this infectious agent as a model to develop and validate the first two steps of a novel diagnostic assay, which includes field sampling and sample processing, based on environmental DNA technology. The environmental DNA technology will be used in conjunction with a targeted approach at first. This involves detecting DNA of a specific pathogen from water samples which will assist in early detection of future disease outbreaks, specifically in South African waters with its rich biodiversity (e.g. 140 odd amphibian species). In doing so, the first two steps of an assay would only require development and validation of one step, should an outbreak of a fungal or fungal-like species currently unknown to us occur.

In conclusion, the new detection assays that are being developed based on eDNA will hopefully assist not only in understanding the current fungal communities, but also assist in building a robust foundation database of host diversity in aquatic ecosystems. Above all and most importantly, this data will assist in predicting outbreaks and hopefully lower the impact of ***ticking bombs*** among our cherished biodiversity.

Ché Weldon

The Anchieta's ridged frog.

APPENDIX B:

Distributional mapping of the aquatic pathogenic fungi of fish and amphibians

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ABSTRACT

Pathogens are a significant conservation concern at a local as well as international scale. Diseases caused by pathogens are a common driver towards permanent as well as temporary population declines. Parasites and diseases have and will always be part of the natural environment. The natural order however has been disrupted due to anthropogenic influences. Human activities have created the means of an increased transmission rate. The main problem however arises from the introduction of pathogens and diseases to areas where they do not naturally occur. *Batrachochytrium dendrobatidis* as well as *Aphanomyces invadans* are to be modelled as they have the potential of becoming a significant conservation threat. *Batrachochytrium dendrobatidis* is an amphibian chytrid fungus that causes chytridiomycosis. The *Batrachochytrium dendrobatidis* pathogen has a wide range of hosts consisting of more than 200 amphibian species globally; this includes various South African amphibian species. *Aphanomyces invadans* is a fungus-like pathogenic oomycete that causes ulcerative mycosis in fish. This ulcerative mycosis causes various skin diseases such as mycotic granulomatosis, epizootic ulcerative syndrome and red spot. Since the initial outbreak in 2007, a large-scale infection has taken place across approximately 22 different species across southern Africa. Habitat suitability models are created for both pathogens using the MaxEnt modelling procedure. The models were generated using 20 environmental parameters and known geo-referenced distributional data found in literature sources. The *Batrachochytrium dendrobatidis* pathogen has a potential widespread distribution throughout South Africa, with the Western Cape and KwaZulu-Natal identified as occurrence hotspots. This is a significant concern as 13 of the 20 most endangered species of amphibians are found in these two regions. *Aphanomyces invadans* has limited areas that are suitable for the pathogen. This result is however due to the poorly researched pathogen. Literature only provided seven locations where the pathogen has been sampled. The model however indicated that the two largest river systems of South Africa are vulnerable due to the known location of the pathogen. One key aspect that was found when generating the models, is that of sampling bias. Sampling bias has a detrimental effect on the models as the true extents of the pathogens are unknown. It is thus recommended that research be done on these pathogens to establish the true extent of the pathogens to develop an effective management plan.

KEYWORDS

Amphibian; *Apanomyces invandans;* Aquatic pathogen; *Batrachochytrium dendrobatidis*; BIOCLIM; Chytrid; GIS; Habitat suitability model; MaxEnt; Species distribution model

Introduction

Pathogens are a significant

conservation concern at a local as well as international scale (Heard *et al.,* 2013). Diseases caused by pathogens are a common driver towards permanent as well as temporary population declines (Heard *et al.,* 2013; De Castro & Bolker, 2005; Pedersen *et al.,* 2007). In the international context, pathogens and their related diseases have a rather limited influence with regards to global extinctions of species (Smith *et al.,* 2006). The effect of pathogens is overshadowed by factors such as invasive species, habitat destruction/loss and overexploitation with these factors causing 45% to 54% of all extinctions (Clavero & Garcia-Berthou, 2005; Hoffmann *et al.,* 2010; Heard *et al.,* 2013). It is however important not to underestimate the potential impact of pathogens on species populations, it might not be a leading cause of animal extinction but in some taxa, it is a leading cause in population declines (Heard *et al.,* 2013). Parasites and diseases have and will always be part of the natural environment, these factors act as an ecological as well as wildlife regulator (Wilson & Primack, 2019). The natural order however has been disrupted due to anthropogenic influences. Human activities have created the means of an increased transmission rate and even conditions leading to epidemics (Wilson & Primack, 2019). The main problem however arises from the introduction of pathogens and diseases to areas where they do not naturally occur (Wilson & Primack, 2019). With this in mind, two pathogens with the potential of becoming a major conservation threat within the context of South Africa are to be explored and a potential distribution map/habitat suitability model is to be created for each species. These two species of pathogens are *Batrachochytrium dendrobatidis* as well as *Aphanomyces invadans,* these pathogens are placed in the introduced diseases category.

Batrachochytrium dendrobatidis (hereafter *Bd*) is an amphibian chytrid fungus that causes chytridiomycosis leading to the death of the host (Olson *et al*., 2013; Boyle *et al*., 2004; Tarrant *et al*., 2013; Piotrowski *et al*., 2004; Longcore *et al*., 1999; Harris *et al*., 2006). The *Bd* pathogen most likely originated on the Korean Peninsula (O'Hanlon *et al*., 2018). A significant portion of amphibians have a threatened status, this is due to various factors that includes the influence of the widespread *Bd* pathogen (Olson *et al*., 2013; Boyle *et al*., 2014; Harris *et al*., 2006; Rachowicz & Vredenburg, 2004; Heard *et al.,* 2013). The *Bd* pathogen has a wide range of hosts consisting of more than 200 amphibian species globally (Tarrant *et al*., 2013; Hirschfeld *et al*., 2016; Wilson & Primack, 2019). This wide host range as well as other factors put significant pressure on a species and can even lead to large scale extinction within the country (Olson *et al*., 2013; Boyle *et al*., 2014). The importance and influence of the *Bd* pathogen has been relatively neglected and has recently been noted as a critical threat towards South African amphibians (Tarrant *et al*., 2013). This creates a data void with regards to the pathogen making potential distribution models/habitat suitability models of great importance with regards to conservation efforts of amphibians.

Aphanomyces invadans (hereafter *Ai*) is a fungus-like pathogenic oomycete that causes ulcerative mycosis in fish (Vandersea *et al*., 2005). The *Ai* pathogen originates from Asia (Huchzermeyer *et al*., 2018). This ulcerative mycosis causes various skin

diseases such as mycotic granulomatosis, epizootic ulcerative syndrome, and red spot (Vandersea *et al*., 2005; Kuan *et al*., 2013; Iberahim *et al.,* 2018). The above-mentioned diseases are detrimental towards the health of the host fish and in many cases even lead to their death (Vandersea *et al*., 2005; Kuan *et al*., 2013). The *Ai* pathogen has been found to infect cultured as well as wild fish (Vandersea *et al*., 2005; Sibanda *et al*., 2018; Huchzermeyer *et al.,* 2018), which are found globally and consists of approximately 94 species (Iberahim *et al.,* 2018). These 94 species show high mortality rates especially with regards to young fish (Vandersea *et al*., 2005; Abbass *et al*., 2004; Youssuf *et al*., 2017; Huchzermeyer *et al.,* 2018; Iberahim *et al.,* 2018). The first outbreak of *Ai* in Africa occurred in the Chobe-Zambezi River (Sibanda *et al*., 2018; Huchzermeyer *et al.,* 2018). Since the initial outbreak in 2007, a large-scale infection has taken place across approximately 22 different species across southern Africa (Sibanda *et al*., 2018; Huchzermeyer *et al.,* 2018; Iberahim *et al.,* 2018). *Ai* has had a very rapid spread in a relatively short period of time and thus has the potential of becoming a significant conservation concern (Baldock *et al.,* 2005; Lilley *et al.,* 1997).

These pathogens and their threat posed to biodiversity in South Africa indicates the importance of monitoring projects aimed at limiting the spread of the disease-causing pathogens. Understanding the working and constraints of a given species' distributional range is however inherently difficult. Distributional modelling serves as an important component towards a better understanding of a species distribution (Kearney & Porter, 2009; Andrewartha & Birch, 1954). This important component is made possible by the advancement of geographic information systems (GIS) software. The GIS software creates the means of creating high quality as well as sophisticated models aimed at establishing possible distributional ranges of any given species, in this case, that of aquatic pathogens (Kearney & Porter, 2009; Peterson, 2001; Scott *et al.*, 1996). The success of GIS modelling is partly due to the understanding of the link between species distribution (abundance) and the environment parameters/conditions (Kearney & Porter, 2009; Guisan & Zimmerman, 2000; Guisan & Thuiller, 2005; Elith *et al*., 2006). It was realized that there is a strong correlation between environmental conditions and distribution. This important realization is due to the combined efforts of zoological as well as geographical fields as well as the advancements in available technologies. A predictive model is created using species locality data that consists of geo-referenced coordinate data (collected in field work/studies) and environmental parameters (Ward, 2007; Anderson *et al*., 2003). The resulting model is projected onto a map and this output is called the habitat suitability model/map (Ward, 2007; Anderson *et al*., 2003). This project thus aims to establish the foundation for a monitoring project through the creation of habitat suitability models. Areas of likely occurrence can become the start for molecular testing as to establish the strain and the spread of the pathogens.

Materials and methods

MaxEnt was used as the predictive modelling software to model the potential distributional range of both *Bd* and *Ai*. MaxEnt was used as it has proven to be an effective as well as popular modelling approach (Gibson *et al.,* 2007; Pearson *et al.,* 2007; Phillips *et al.,* 2006; Hernandez *et al.,* 2008; Booth *et al.,* 2014; Halvorsen *et al.,* 2015). MaxEnt uses presence-only distributional data as basis for model generation along with climate data (Elith *et al.,* 2011).

Bd distribution data

The positional data used in the predictive model was obtained from various sources. These included:

Conradie, W., Weldon, C., Smith, K.G. & Du Preez, L.H. 2011. Seasonal pattern of chytridiomycosis in common river frog (Amietia angolensis) tadpoles in the South African Grassland Biome. *African Zoology*, 46(1):95-102.

Hopkins, S. & Channing, A. 2003. Chytrid fungus in northern and western cape frog populations, South Africa. *Herpetological Review*, 34(4):334.

Lane, E., Weldon, C. & Bingham, J. 2003. Histological evidence of chytridiomycete fungal infection in a free-ranging amphibian, Afrana fuscigula (Anura: Ranidae), in South Africa. *Journal of the South African Veterinary Association*, 74(1):20-21.

Smith, K.G., Weldon, C., Conradie, W. & du Preez, L.H. 2007. Relationships among size, development, and Batrachochytrium dendrobatidis infection in African tadpoles. *Diseases of aquatic organisms*, 74(2):159-164.

Tarrant, J., Cilliers, D., Du Preez, L.H. & Weldon, C. 2013. Spatial assessment of amphibian chytrid fungus (*Batrachochytrium dendrobatidis*) in South Africa confirms endemic and widespread infection. *PloS One*, 8(7).

Vredenburg, V.T., Felt, S.A., Morgan, E.C., McNally, S.V., Wilson, S. & Green, S.L. 2013. Prevalence of Batrachochytrium dendrobatidis in Xenopus collected in Africa (1871-2000) and in California (2001-2010). *PLoS One*, 8(5): e63791.

Weldon, C. 2005. Chytridiomycosis, an emerging infectious disease of amphibians in South Africa. North-West University.

The sources provided a total of 60 presence data points. This was sufficient as this meets the required minimum of 40, for generating a habitat suitability model. The location of these data points can be seen in Figure 1.

Ai distribution data

The positional data used in the predictive model was obtained from various sources. These are:

Huchzermeyer, C., Huchzermeyer, K., Christison, K., Macey, B., Colly, P., Hang'ombe, B. & Songe, M. 2018. First record of epizootic ulcerative syndrome from the Upper Congo catchment: An outbreak in the Bangweulu swamps, Zambia. *Journal of fish diseases*, 41(1):87-94.

Iberahim, N.A., Trusch, F. & Van West, P. 2018. Aphanomyces invadans, the causal agent of Epizootic Ulcerative Syndrome, is a global threat to wild and farmed fish. *Fungal Biology Reviews*, 32(3):118-130.

Kajee, M., Griffiths, C. & Lamberth, S. 2018. Long-term physico-chemical and faunal changes in a small, rural South African estuary. *African Zoology*, 53(4):127-137.

Malherbe, W., Christison, K., Wepener, V. & Smit, N. 2019. Epizootic ulcerative syndrome – First report of evidence from South Africa's largest and premier conservation area, the Kruger National Park. *International Journal for Parasitology: Parasites and Wildlife*, 10:207-210.

From the outset it was clear that research regarding the South African distribution of *Ai* was limited and incomplete. Due to this, a different approach was required in establishing the presence data for the habitat suitability model. A total of seven sites were identified where *Ai* has been documented within the borders of South Africa, this is below the minimum data points as recommended by MaxEnt software. To increase the data points to the recommended quantity, each site was given a total of six data points. Dams and lakes were given six evenly distributed data points that covered the entire extent of the water body. Rivers presented a challenge due to overall length. Areas where samples were located were roughly indicated in the literature. Two data points were placed near where the sample was located and identified (based on a literature description) the remaining four data points were placed in even intervals from the “base points”, two upstream and two downstream. This procedure (for rivers and dams) increased the data point count from seven to 42. The geo-referenced locational data of *Ai* as well as *Bd* can be seen in Figure 1.

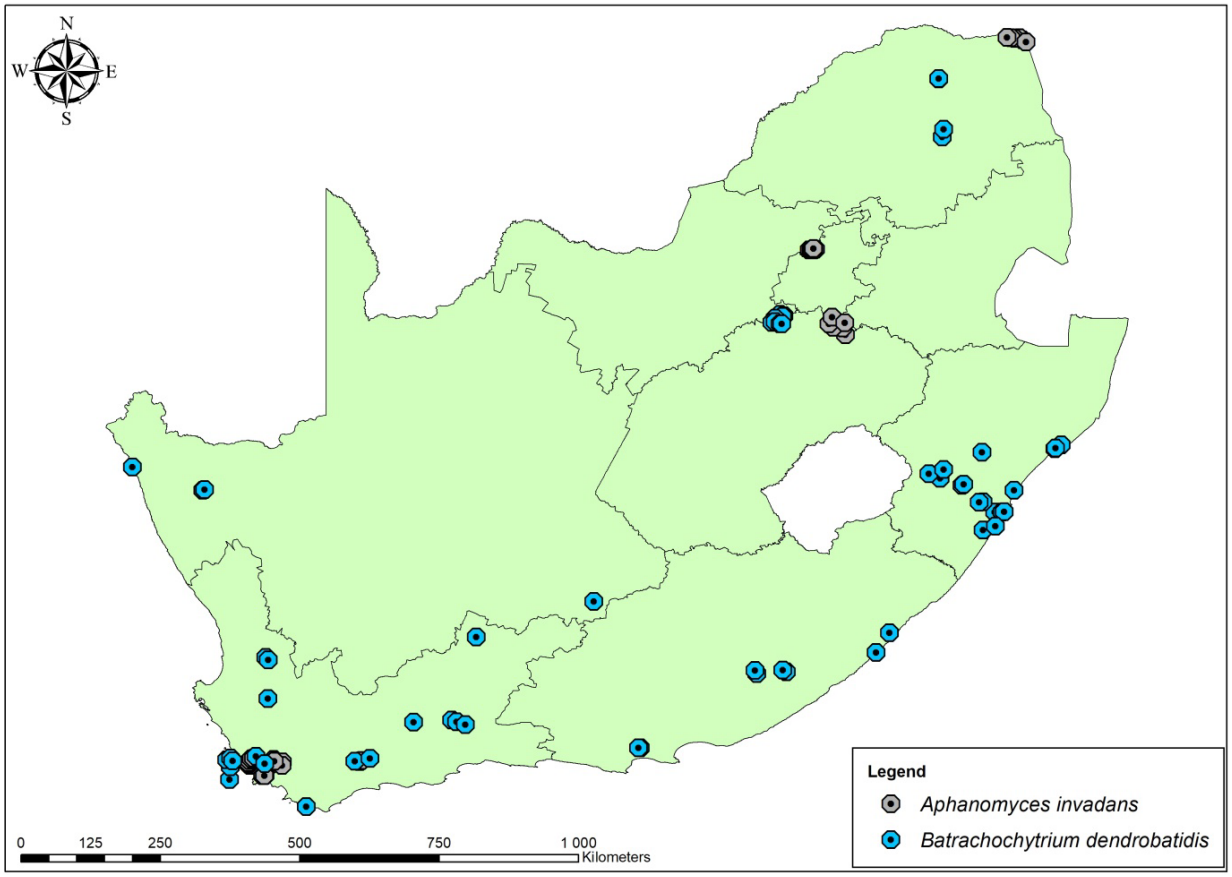


Figure 1: The presence data points of *Aphanomyces invadans* (42) and *Batrachochytrium dendrobatidis* (60) that was used in generating the habitat suitability model.

The environmental variables that were used in generating the potential distribution models consisted of bioclimatic variables obtained from BIOCLIM (WorldClim, 2020). In addition to the 19 standard bioclimatic variables, an elevation dataset was also included (also obtained from the same source). Table 1 indicates the 20 BIOCLIM layers used. The bioclimatic as well as elevation datasets consist of pixels, with each pixel being the size of one square kilometre. It was decided not to re-sample the data. This decision was based on the geographic extent of the modelled area. The default model parameters were used in model generation. The generated outputs of the MaxEnt procedure consisted of response curves, prediction pictures (models) as well as a jack-knife that measures the importance of each variable. These outputs are key as they identify the variables that are deemed to have the greatest contribution to the given species expected distributional range.

Table 1: The variables used in creating the potential distribution model for *Bd* and *Ai* (WorldClim, 2020).

Layer Name	Layer description
Bio01	The annual Mean Temperature
Bio02	The mean Diurnal Range (Mean of monthly (max temp - min temp)
Bio03	The isothermality (BIO2/BIO7) (×100)
Bio04	The temperature Seasonality (standard deviation ×100)
Bio05	The max Temperature of Warmest Month
Bio06	The min Temperature of Coldest Month
Bio07	The temperature Annual Range (BIO5-BIO6)
Bio08	The mean Temperature of Wettest Quarter
Bio09	The mean Temperature of Driest Quarter
Bio10	The mean Temperature of Warmest Quarter
Bio11	The mean Temperature of Coldest Quarter
Bio12	The annual Precipitation
Bio13	The precipitation of Wettest Month
Bio14	The precipitation of Driest Month
Bio15	The precipitation Seasonality (Coefficient of Variation)
Bio16	The precipitation of Wettest Quarter
Bio17	The precipitation of Driest Quarter
Bio18	The precipitation of Warmest Quarter
Bio19	The precipitation of Coldest Quarter
Elevation	The height above sea-level

The presence-only data and climate variables were thus used in conjunction to generate the habitat suitability model for each pathogen species.

Results

Bd

The resulting habitat suitability model for *Bd* can be seen in Figure 2. The model was generated using 60 positional data points along with the BioClim variables. As seen in the figure, the *Bd* pathogen has a wide potential distribution with likely occurrence in each province. It is however clear that there are two hotspots with a significant range. These are the southern part of KwaZulu-Natal and the majority of the Western Cape.

Figure 3 indicates the habitat suitability model of *Bd* as well as the positional data of two of the pathogens known hosts (*Amietia delalandii* and *Amietia fuscigula)*. The data points can be grouped into two broad regions. The coastal belt (Western Cape, Eastern Cape, and KwaZulu-Natal) and the central band (Gauteng, Mpumalanga and Limpopo).

The Jack-knife test indicated (as seen in Table 2) that Bio02 (The mean Diurnal Range), Bio19 (The precipitation of Coldest Quarter), Bio05 (The max Temperature of

Warmest Month) and Elevation have the largest impact of the generated model. The other variables have little to no effect on the model.

Table 2: The Jack-knife of the habitat suitability model of *Bd*

Variable	Percent contribution (%)
Bio02	33.2
Bio19	30.8
Bio05	19.2
Elevation	6.8
Bio12	1.9
Bio03	1.8
Bio13	1.7
Bio06	1.6
Bio14	1.0
Bio04	0.8
Bio17	0.5
Bio18	0.3
Bio11	0.2
Bio15	0.1
Bio08	0.1
Bio10	0.0
Bio01	0.0
Bio07	0.0
Bio16	0.0
Bio09	0.0
Total	100.0

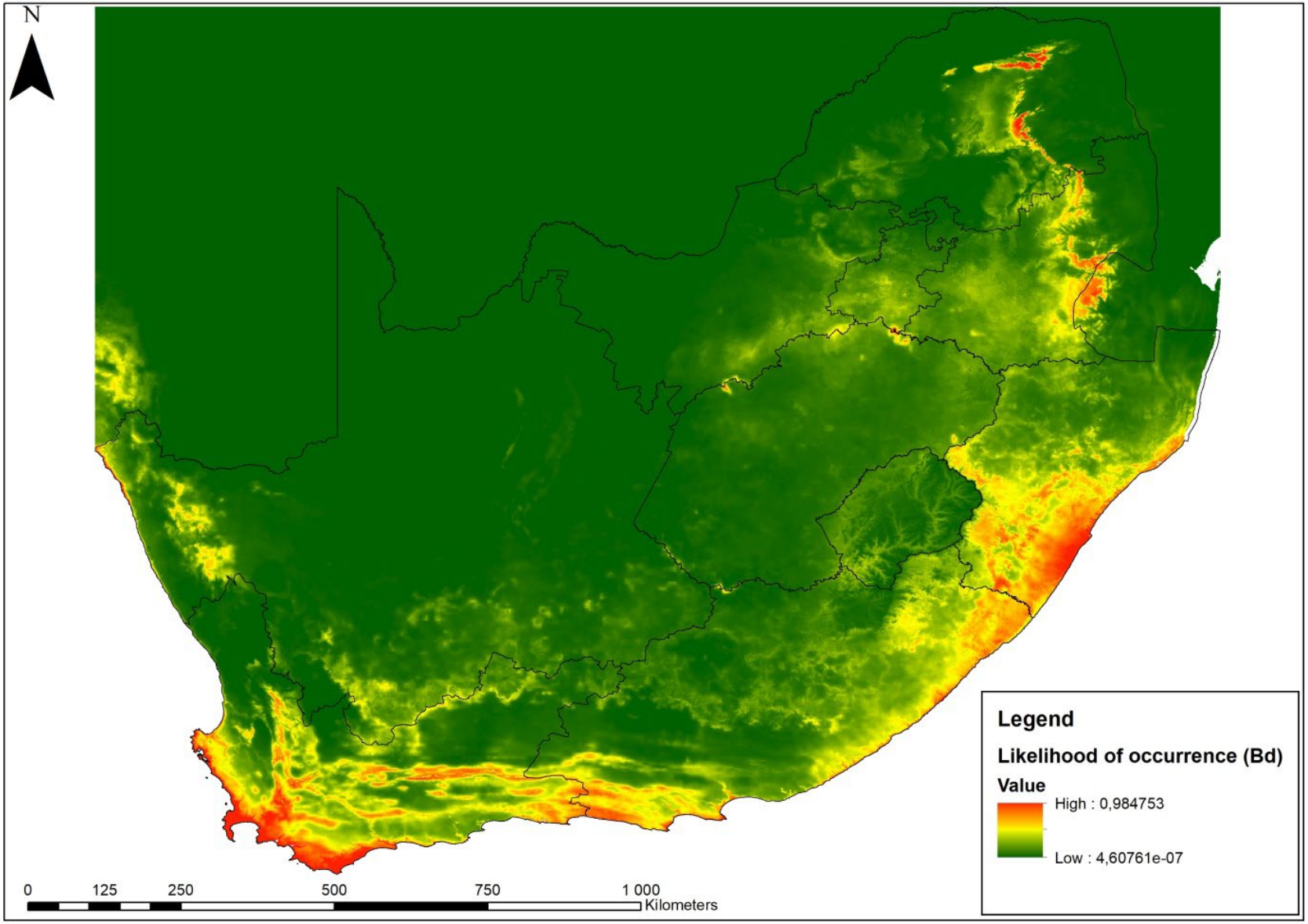
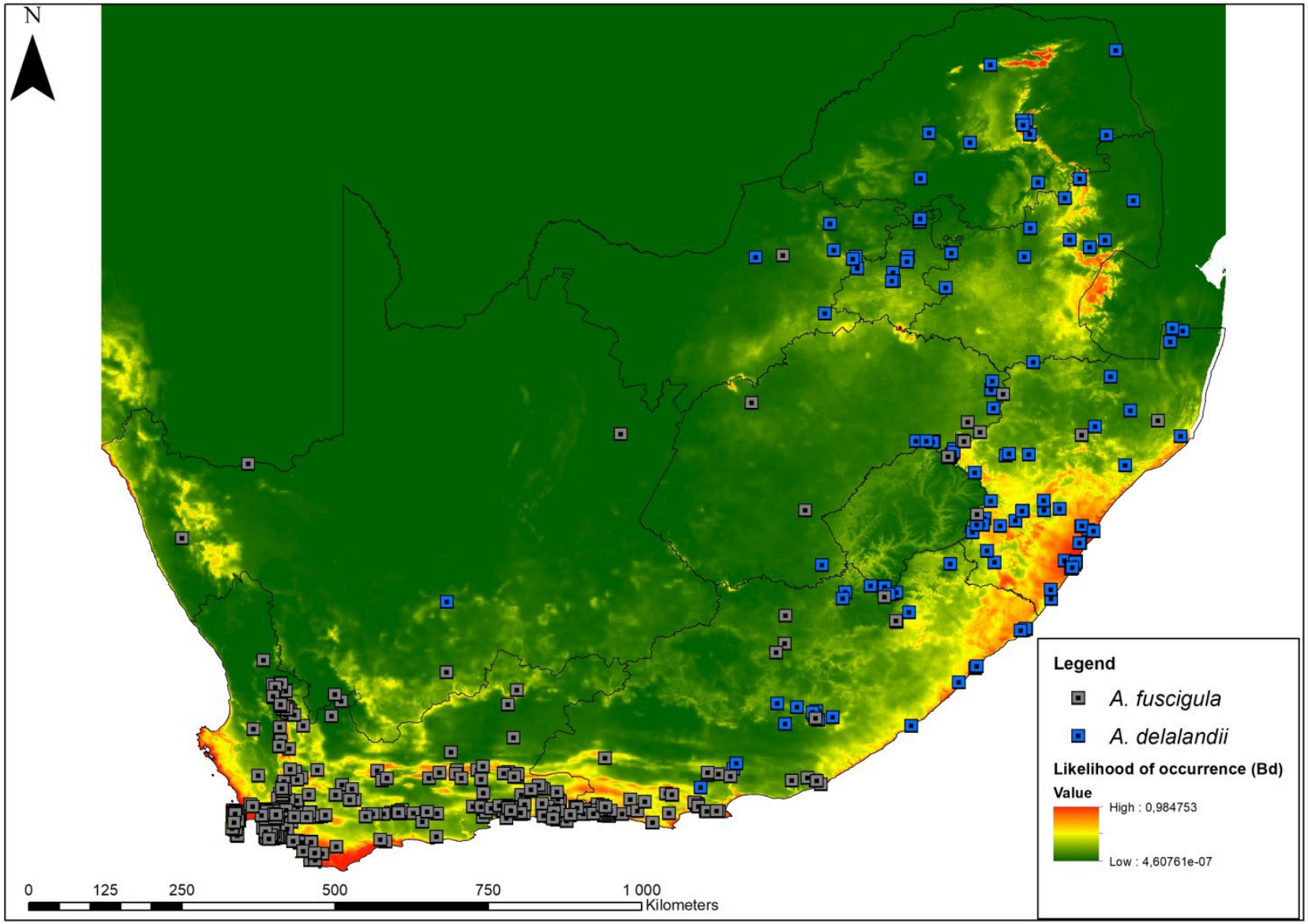


Figure 2: The habitat suitability model of *Batrachochytrium dendrobatidis.*


N
0
125
250
500
750
1 000
Kilometers
Legend
A. fuscigula
A. delalandii
Likelihood of occurrence (Bd)
Value
High : 0,984753
Low : 4,60761e-07

Ai

The habitat suitability model generated for *Ai* can be seen in Figure 4. The model was generated using 42 positional data points with the BioClim variables. As seen in the figure the *Ai* pathogen is indicated to have a high likelihood of occurrence in central Gauteng, western and northern Limpopo as well as a large part of the Western Cape. There are various other areas where a high likelihood of occurrence exists, but these are limited in size.

Figure 5 indicates the habitat suitability model for *Ai* as well as the positional data points of a known host (*Clarias gariepinus*). The data can be summarized into two broad regions. The first being the coastal belt (Western Cape) and secondly the Central belt (Limpopo, Mpumalanga, and Gauteng).

The Jacknife test indicates (as seen in Table 3) that Bio19 (precipitation of coldest quarter), Bio08 (mean temperature of wettest quarter), Bio02 (mean diurnal range) and Bio03 (isothermality) have the largest influence on the generated habitat suitability model. The other variables have little (less than 6%) to no effect on the habitat suitability model.

Table 3: The Jack-knife of the habitat suitability model of *Ai*

Variable	Percent contribution (%)
Bio19	63.1
Bio08	12.2
Bio02	7.9
Bio03	6.6
Elevation	3.6
Bio14	1.9
Bio12	1.3
Bio01	1.1
Bio04	0.6
Bio16	0.4
Bio17	0.4
Bio05	0.3
Bio07	0.2
Bio15	0.1
Bio06	0.1
Bio18	0.1
Bio09	0.0
Bio10	0.0
Bio11	0.0
Bio13	0.0
Total	100.0

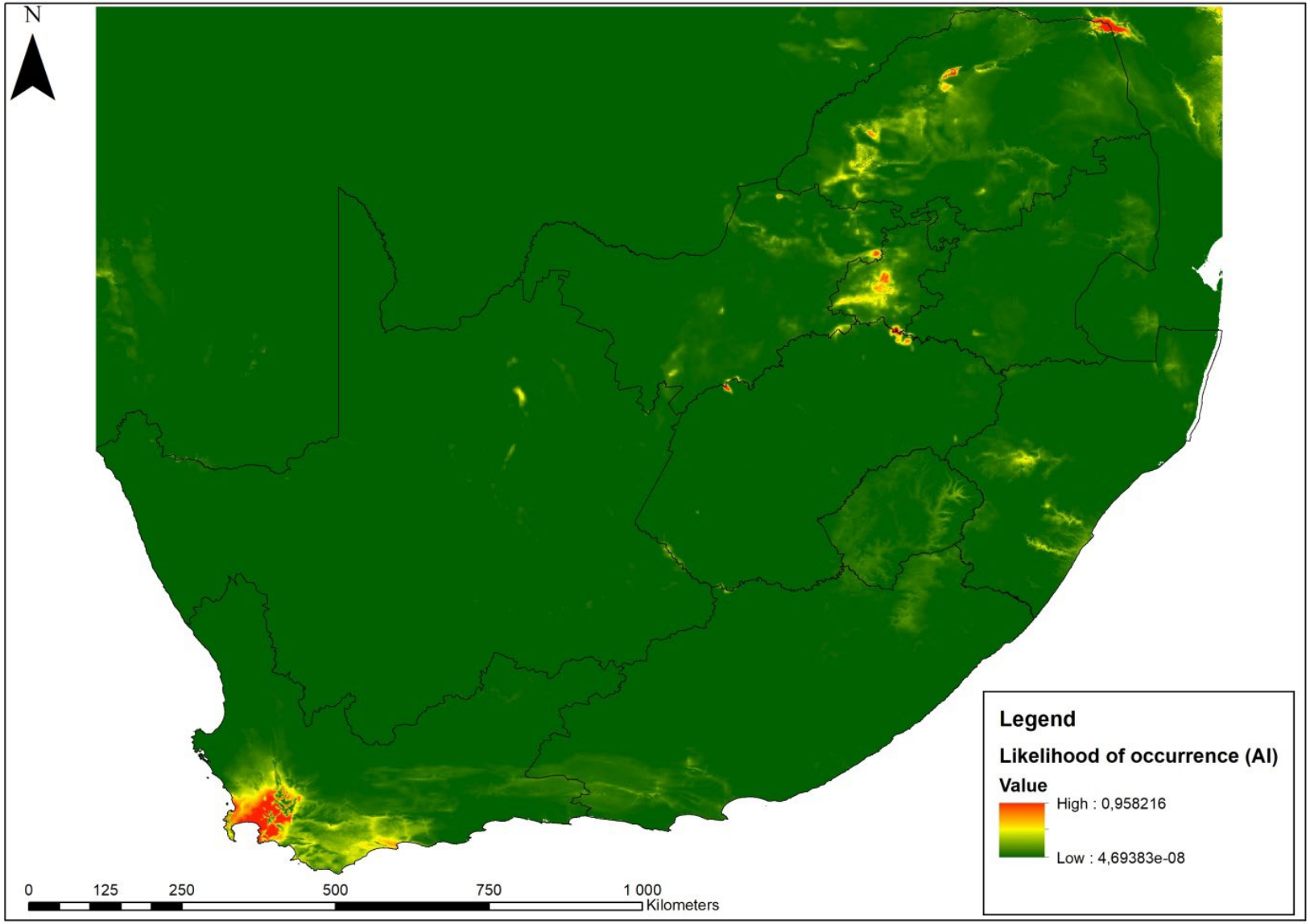


Figure 4: The habitat suitability model of *Aphanomyces invadans.*

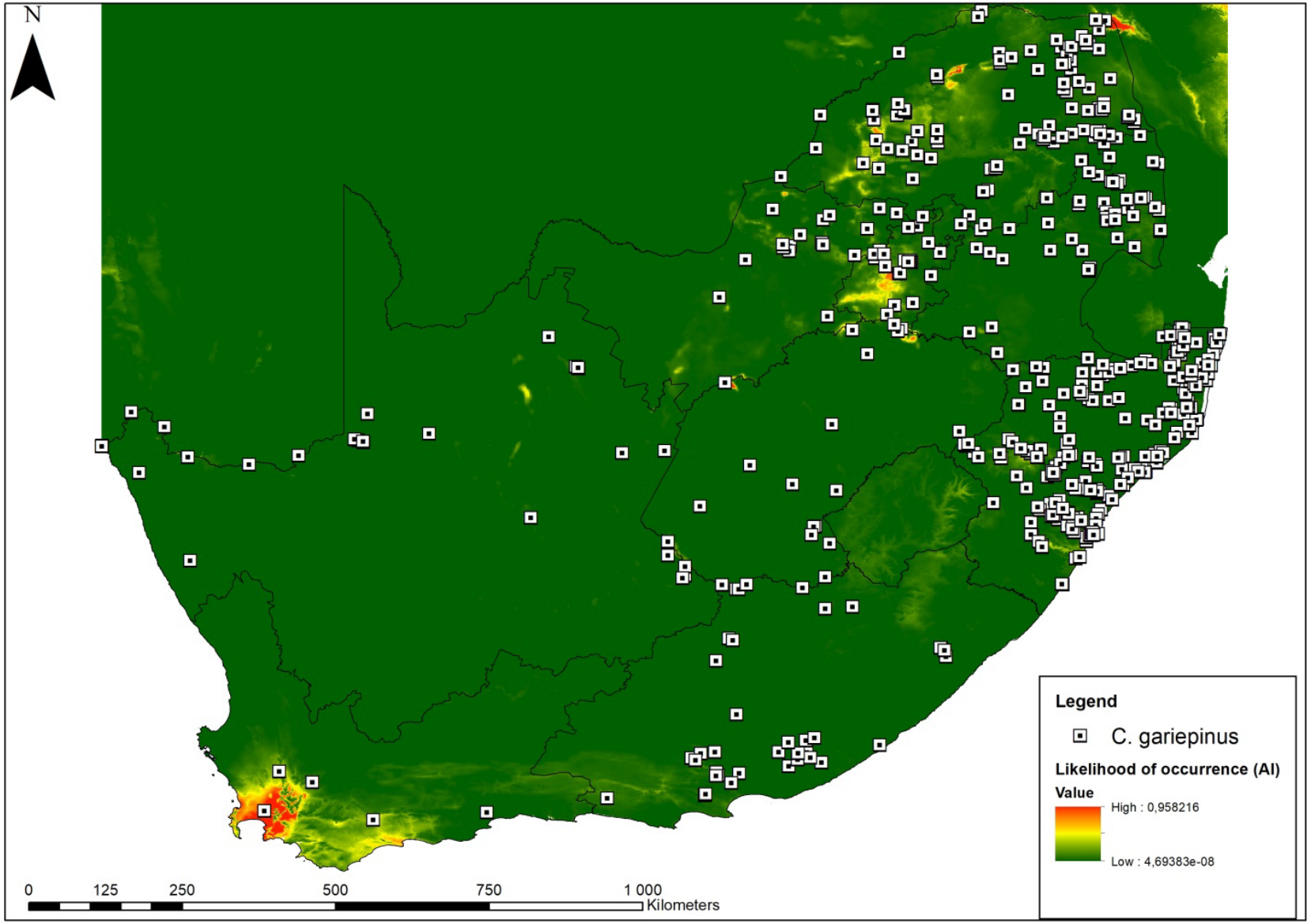


Figure 5: The habitat suitability model of *Aphanomyces invadans* as well as the location of a known host.

Discussion

Bd

The identified *Bd* hotspots of the Western Cape and KwaZulu-Natal present a significant conservation concern. Of the 20 most endangered amphibian species (endangered, critically endangered and vulnerable) of South Africa, 13 are located within these two provinces (Du Preez & Carruthers, 2009).

As seen in Figure 6, seven of the endangered species are distributed throughout the province of KwaZulu-Natal. These are the Natal Leaf-Folding Frog (*Afrixalus spinifrons*), Spotted Shovel-nosed Frog (*Hemisus guttatus*), Knysna Leaf-folding Frog (*Afrixalus knysnae*), Pickersgill's Reed Frog (*Hyperolius pickersgilli*), Long-toed Tree Frog (*Leptopelis xenodactylus*), Kloof Frog (*Natalobatrachus bonebergi*) and the Mistbelt Chirping Frog (*Anhydrophryne ngongoniensis*).

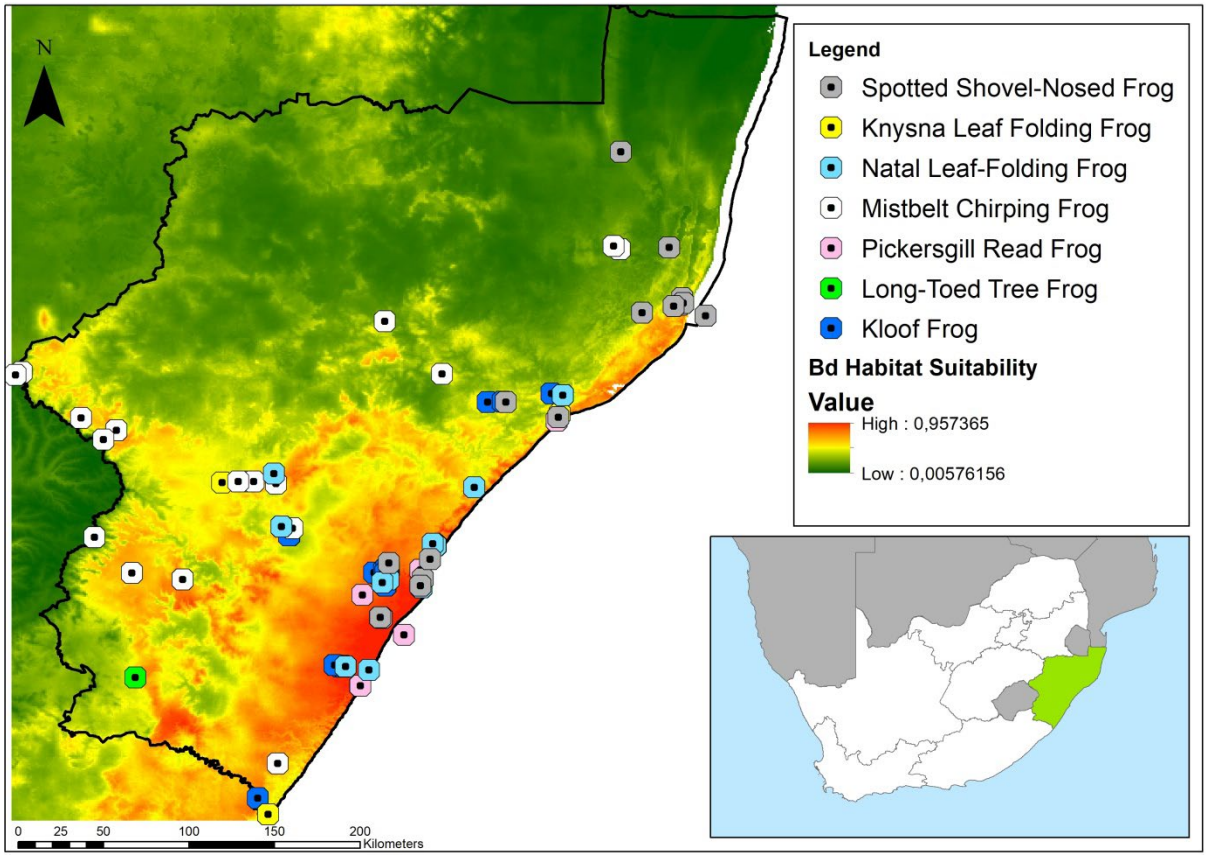


Figure 6: The positional data points of endangered amphibians located in the KwaZulu-Natal province of South Africa.

As seen in Figure 7, nine endangered species occur within the Western Cape Province. These are the Natal Leaf-Folding Frog (*Afrixalus spinifrons*), Cape Rain Frog (*Breviceps gibbosus*), Cape Caco (*Cacosternum capense*), Rose's Mountain Toadlet (*Capensibufo rosei*), Knysna Leaf-folding Frog (*Afrixalus knysnae*), Kloof Frog (*Natalobatrachus bonebergi*), Cape Platanna (*Xenopus gilli*), Table Mountain Ghost Frog (*Heleophryne rosei*) and lastly the Micro Frog (*Microbatrachella capensis*).

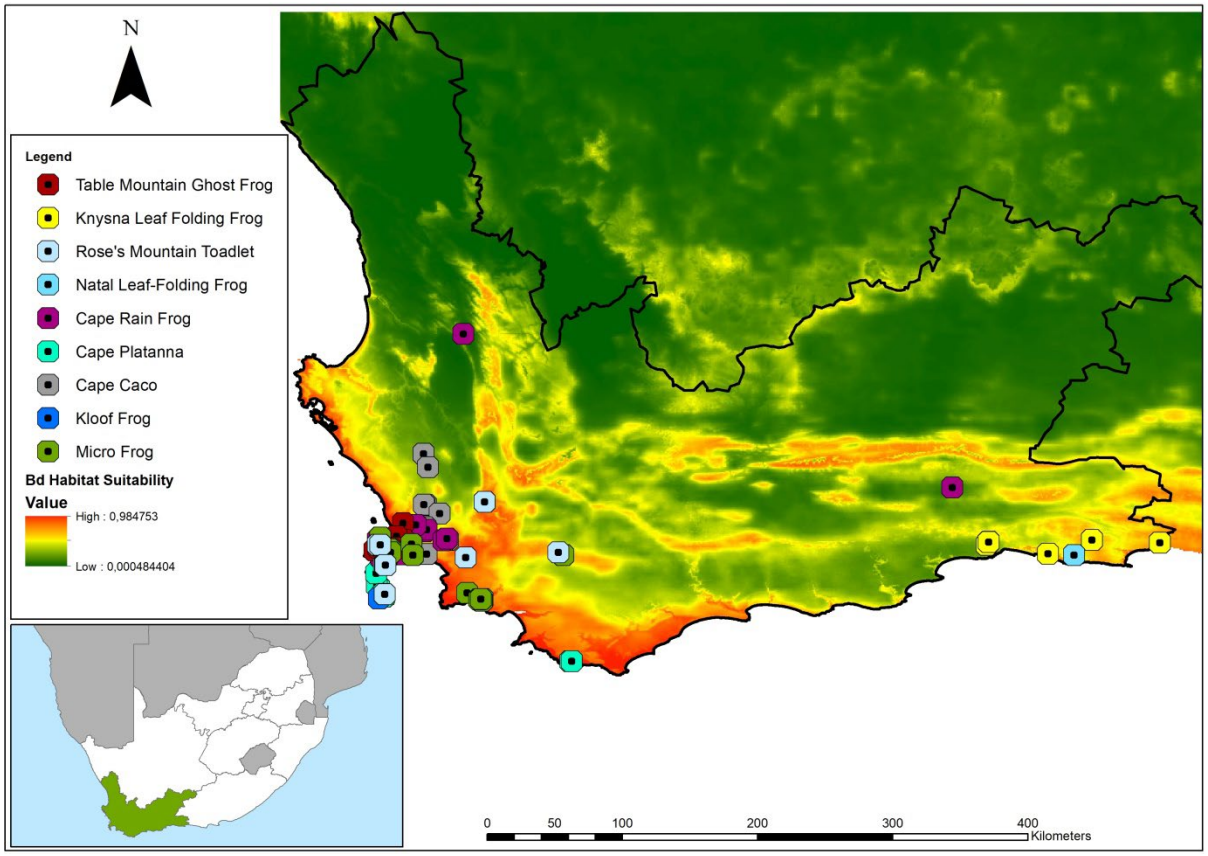


Figure 7: The positional data points of the endangered amphibian's species located in the Western Cape Province of South Africa.

When viewing these two examples of *Bd* hotspots, it is clear that managing and controlling the spread of the *Bd* pathogen is of the utmost importance as this pathogen could have a devastating effect on the endangered amphibians of South Africa.

Ai

When viewing the habitat suitability model of *Ai*, one could be forgiven for thinking that the locations are isolated and contained. The reality however is that it is far from the truth. The coastal zone with the highest likelihood of occurrence and known locations of the pathogen are mostly located in the Western Cape. The areas of high likelihood of occurrence in the central zone (Gauteng, Limpopo, Mpumalanga and the Free State) are within the Limpopo as well as the Vaal/Orange River systems. This is significant as these are the two largest catchments in South Africa (Cambray *et al.,* 1986; Zhu & Ringler, 2012).

The Limpopo River Basin consists of an interconnected system that covers approximately 412 000 km^2 (Zhu & Ringler, 2012). The Limpopo River Basin flows through four countries; these are Botswana, Mozambique, South Africa as well as Zimbabwe (Zhu & Ringler, 2012). The Limpopo River system originates partly in Gauteng where a very high likelihood of *Ai* occurrence exists, as seen in Figure 8. Furthermore, when viewing Figure 8, it can be seen that there is high likelihood of occurrence downstream as well. From this it can thus be assumed that a very significant portion of the river has been compromised as three known *Ai* locations are within the Limpopo River system and hosts are distributed throughout the system. This remains an assumption as further testing is required as to establish the true

extent of the pathogen within the system. It is however clear that this is an international concern as the system spans four countries.

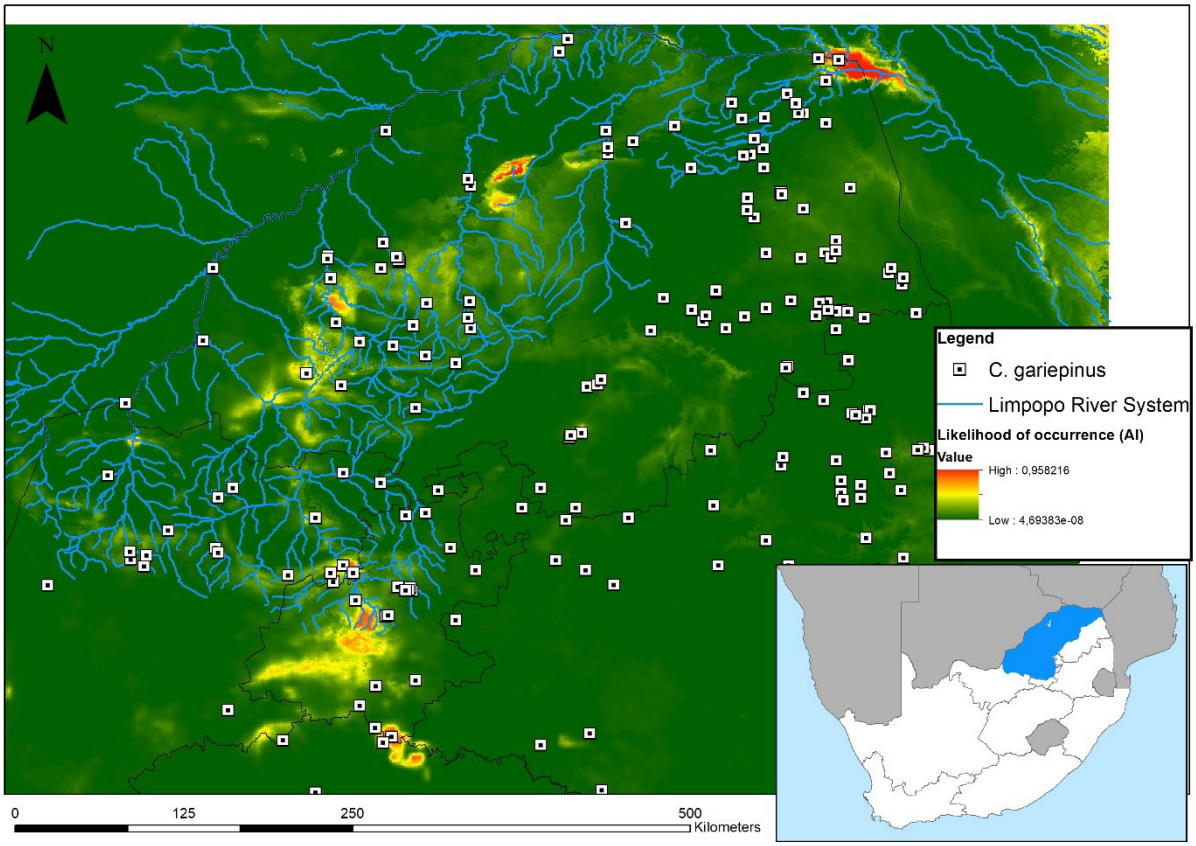


Figure 8: The Limpopo River system and the location of areas high Ai likelihood as well as host locations.

The Orange River system is the largest of South Africa and covers approximately 650 000 km^2 (Cambray *et al.,* 1986). The interconnected system is transboundary as it flows through four countries. These are Lesotho, South Africa, Namibia as well as Botswana (Voetdijk, 2020). A part of the Orange River system originates in Gauteng and the North-West, as seen in Figure 9. This is of importance as the area has been indicated as a possible vulnerable area. Additionally, two of the confirmed *Ai* locations are within the system. This presents a significant concern as one of the identified hosts is located within the Orange River system, as seen in Figure 9. As with the Limpopo River system, this is more than a national concern as four countries are vulnerable.

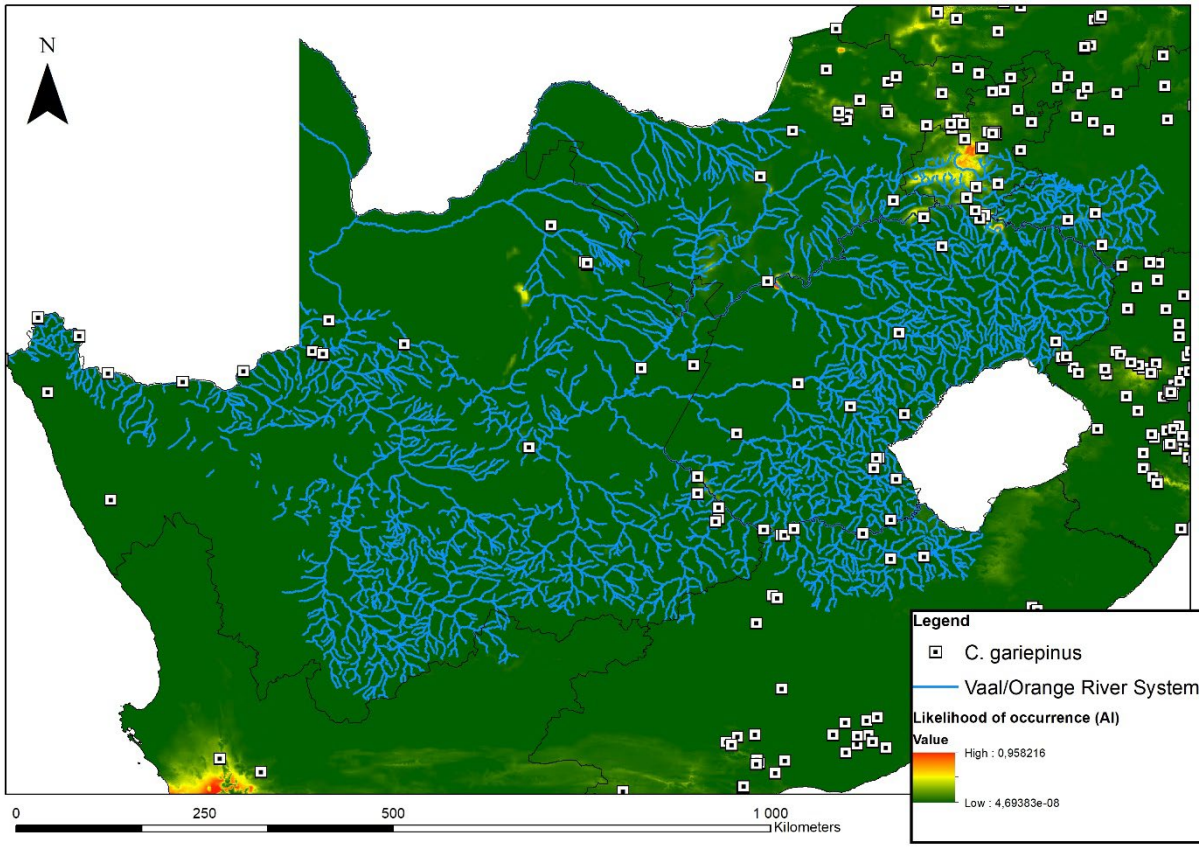


Figure 9: The Orange/Vaal River system and the location of areas high Ai likelihood as well as host locations.

It is clear that the distribution of *Ai* poses not only a potential conservation concern to South Africa, but to neighbouring countries as well. It is thus recommended that further studies be undertaken as to establish the true extent of the pathogen as this model has illustrated the potential danger of the pathogen for southern Africa. It is with this recommendation however that a key disadvantage has been identified with regards to the models of both pathogens.

From the generated models, a potential problem has been identified that limits the effectiveness of potential management plans. The fact that biological records originate from various sources such as systematic population monitoring, surveys and mass public participation has advantages as well as disadvantages (Isaac & Pocock, 2015; August *et al.,* 2015; Pocock *et al.,* 2015). The key advantages are that biological records are of scientific importance and records gathered from various sources greatly increase the scope of a project (Pocock *et al.,* 2015; Isaac & Pocock, 2015). The disadvantage however is that these types of data contain biases and inherit limitations (Bird *et al.,* 2014). It is this concept of biased data that has a large influence on the generated models of *Bd* as well as *Ai*.

The reality is that the existence of bias in biological data and its possible origins of biased data are well known (Prendergast *et al.,* 1993; Isaac & Pocock, 2015). One key source of biased data is due to uneven spatial coverage of sampling (Isaac *et al.,* 2014). Researchers and public records are usually made within a well-defined geographic area (Isaac & Pocock, 2015). In the case of researchers, they tend to do research in areas that have a high diversity

in the taxon of their interest (Prendergast *et al.,* 1993). In the case of public participation, biased data can originate simply due to the fact that the records are made close to where they live (Isaac & Pocock, 2015). It is thus assumed that the largest concentration of records is to be found near large populations and favoured research areas.

Conclusion

The *Ai* and *Bd* pathogen present a significant threat to the biodiversity not only in South Africa, but internationally. The habitat suitability models have indicated "hotspots" where the occurrence of the pathogens is almost certain. These "hotspots" present challenges as they are either in an area with high diversity and vulnerability or areas with an interconnected nature spanning various habitats and countries. The procedure has identified various disadvantages and factors that limit the accuracy of the generated models. The records used in creating the host as well as pathogen distribution maps are presence only data, this means that there is a high likelihood that there are areas that have either the host or the pathogen but due to biased collection they are not indicated. This is reflected in the potential distribution model of *Bd* as the areas with the highest concentration of records are located in the provinces of Kwa-Zulu Natal and the Western Cape. Both these areas are highly diverse and have large populations. Furthermore, when consulting the host distribution map, the same pattern appears. It is thus impossible to indicate the true available niche of both pathogens. It is clear that more research is required with regards to the pathogens with specific emphasis on national distribution. Additional data points will aid in creating more accurate models that provide a better foundation for any management plan targeted at the pathogens.

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